Document Title

Tier 2 Summary of the Toxicological and Toxicokinetic Studies of the Active Substance, Flupyradifurone TC

Specification No.:

102000025488

Data Requirements

Manual of Requirements and Guidelines, 4th edition/APVMA/Australia
Decree 4.074/2002/Brazil
Regulatory Directive 2003-01/Canada/PMRA
Regulation (EC) No 1107/2009
REGULATION in Matter of Registration, Import and Export Authorization and Export Certificates for Pesticides, Nutrients for Plants and Toxic or Dangerous Substances and Materials//SSA-SAG/ARPA-SEMARNAT/Mexico
US EPA OCSPP Guideline Number SUPP

Annex II
Section 3, Point 5
Document M

According to OECD format guidance for industry data submissions on plant protection products and their active substances

Laboratory Testing Facility

Not Applicable

Date

2014-11-20

Author(s)

[redacted], J.; [redacted] D.; [redacted]; [redacted], O., [redacted], B.

Bayer CropScience

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Company: Bayer CropScience AG

Company Agent: [redacted]

Title: Regulatory Manager

Date: August 6, 2012

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GOOD LABORATORY PRACTICE STATEMENT

This report contains information that is descriptive in nature and is not an actual study, and therefore is not subject to the requirements of GLP under 40 CFR 160.

SPONSOR/SUBMITTER

Bayer CropScience AG

Name: [redacted]

Date: August 6, 2012

STUDY DIRECTOR

As this document is not subject to the requirements of GLP, there is no study director.

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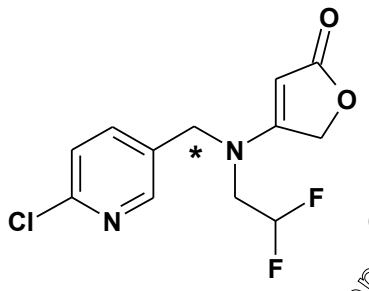
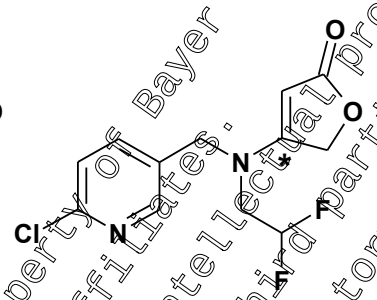
KIIA 5 - Toxicological and Toxicokinetic Studies on the Active Substance

Executive Summary

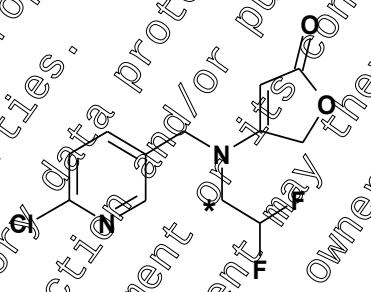
Absorption, distribution, excretion and metabolism of the new insecticide BYI 02960 (common name flupyradifurone) was investigated using three different labelling positions. The active substance was labelled with ^{14}C in the pyridinylmethylene bridge, in the 4-position of the furanone ring and in the 1-position of the ethyl side chain:



Pyridinylmethyl-^{14}C



Furanone-4-^{14}C



Ethyl-1-^{14}C

The pyridinylmethyl-labelled compound was used in an ADME-study in which male and female rats were orally administered with a low dose of 2 mg/kg and a high dose of 200 mg/kg. Due to the high water solubility of BYI 02960, male rats were also given an intravenous dose of 2 mg/kg. In this study, the excretion via urine and faeces was investigated as well as the distribution in the plasma and the radioactivity concentration in organs and tissues at sacrifice. The metabolism was investigated in urine and faeces.

A quantitative whole body autoradiography study was conducted also using the pyridinylmethyl-labelled compound following a single oral dose of 5 mg/kg to male and female rats. In this study the excretion of radioactivity was determined in urine, faeces and expired air as well as the distribution in the plasma and the radioactivity concentration in the organs and tissues at various time points.
The furanone-4-labelled compound was used in an ADME-study in which male and female rats were orally administered with 2 mg/kg. In this study, the excretion via urine and faeces was investigated as well as the distribution in the plasma and the radioactivity concentration in the organs and tissues at sacrifice. The metabolism was investigated in urine and faeces.
A quantitative whole body autoradiography study was also conducted using the furanone-4-labelled compound following a single oral dose of 5 mg/kg to male and female rats. In this study the excretion of radioactivity was determined in urine, faeces and the expired air as well as the distribution in the plasma and the radioactivity concentration in the organs and tissues at various time points.
In an organ metabolism study, male and female rats were orally administered with a single dose of 3 mg/kg [furanone-4-^{14}C]BYI 02960. Animals were sacrificed 6 h after dosage and the metabolism was investigated in urine, plasma, and in extracts of liver, kidney muscle and fat.

The ethyl-1-labelled compound was used in an ADME-study in which male rats were orally administered with 2 mg/kg. In this study, the excretion via urine, faeces and expired air was

investigated as well as the distribution in the plasma and the radioactivity concentration in the organs and tissues at sacrifice. The metabolism was investigated in urine and faeces.
In an organ metabolism study, male and female rats were orally administered with a single dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. The animals were sacrificed 1 h, 6 h, and 24 h after dosing. The total radioactivity was determined at different time points in urine, while in plasma, liver, kidney, muscle and fat at sacrifice. The metabolism was investigated in urine, plasma, and in extracts of liver, kidney, muscle, and fat.

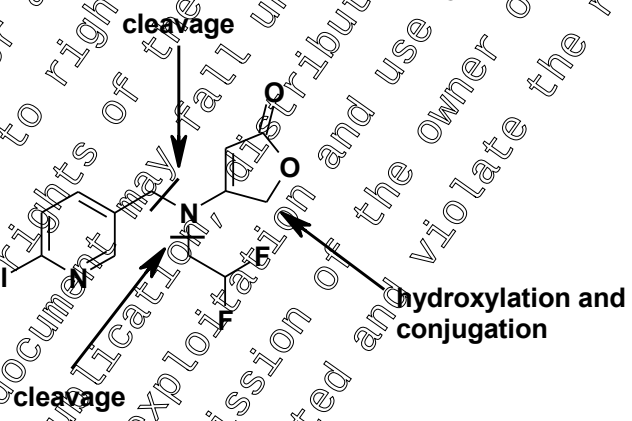
Following oral administration of a low dose of BYI 02960 to male and female rats, the gastrointestinal absorption of radioactivity was high. It accounted for ≥80 % of the dose independent of the labelling position used. Excretion was very fast, mainly renal and almost completed after 24 h. No radioactivity was detected in the expired air after dosing of the pyridinylmethyl- and ethyl-1-labelled compounds, proving the stability of these labelling positions in the molecule. Only after administration of [furanone-4-^{14}C]BYI 02960 between 1 and 3% of the administered radioactivity was exhaled. This demonstrated that for a small portion of the dose (higher in males than in females) the furanone ring of the molecule obviously was opened and underwent biotransformation to C-1 fragments.
The maximum plasma concentration was reached in most cases within 1 or 2 hours after administration of low doses. Only after administration of the high dose the peak plasma concentration was observed between 2 and 4 hours after dosage. After reaching the peak concentration, the radioactivity levels in plasma declined steadily by several orders of magnitude in all studies independent of sex or labelling position of the test compound.

Quantitative whole body autoradiography revealed a fast absorption and distribution of the test compound with peak values observed already 1 h after administration. At this time, the concentrations in liver and kidney were significantly higher than in blood, suggesting a preferred clearance from blood and distribution mainly to these organs which are mainly responsible for metabolism (liver) and excretion (kidney). Higher levels than in blood were also detected in the myocardium (heart) and in several glands. Very low levels were found in the brain, spinal cord and renal fat. These results are similar in male and female rats independent of the labelling position. A fast decline of radioactivity concentrations was observed for all organs and tissues in males and females during the entire test period. Concentrations fell for most organs and tissues below 5% of the maximum after one day. After seven days, only very low concentrations were found in a few organs and tissues of rats dosed with the pyridinylmethyl-labelled test compound. In the study using the furanone-4-labelled compound, low radioactive residues were measured in almost all organs and tissues due to the incorporation of C1- or C2-fragments into the endogenous carbon pool. The residues in males were higher by a factor of 1.4 to 4.7 as compared to females. A similar ratio of approx. 3 (males/females) was also found for the formation of $^{14}CO_2$. This is presumably due to sex related differences in metabolism leading to more C1- and C2-fragments and also higher incorporation of these components into the endogenous carbon pool in male rats. Basically males and female rats exhibited a very similar absorption, distribution and excretion behaviour. The results of these studies demonstrate that there is no indication of any accumulation or significant retention of radioactivity in male and female rats. This observation is supported by the low P_{ow} of 1.2. Concentrations of radioactivity detected in tissues and organs at sacrifice were either very low or below the limit of detection.

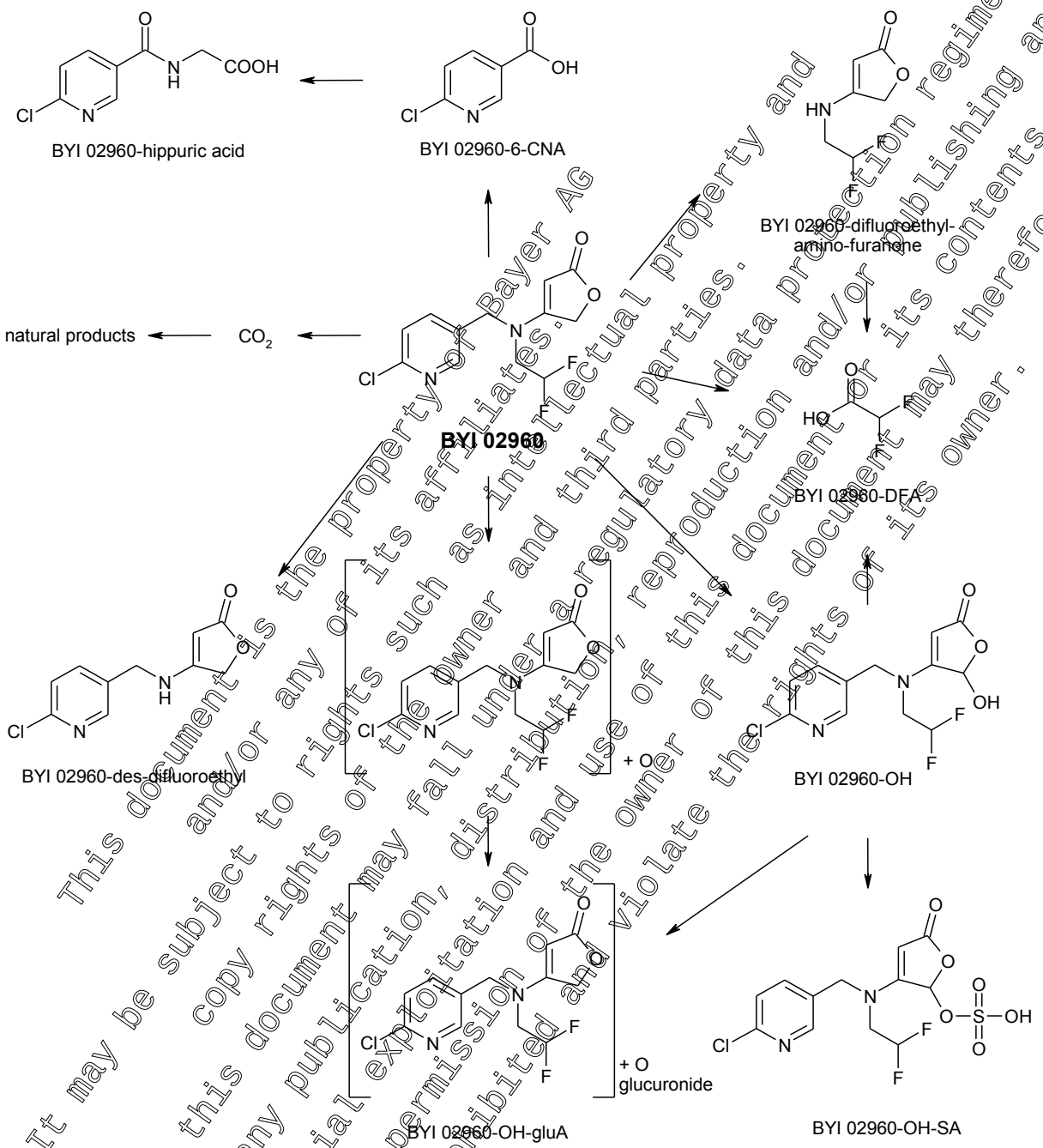
BYI 02960 was intensively metabolised in the rat. Numerous metabolites were formed, most of them being minor ones. The parent compound represented the predominant part of the radioactivity in urine of male and female rats. In faeces of male rats, the metabolite BYI 02960-OH was more prominent than the parent compound. Two metabolites, BYI 02960-6-CNA and BYI 02960-hippuric acid were also prominent in male but not in females rats.
The organ metabolism study using the ethyl-1-^{14}C label showed that in the 24 hours samples of plasma, and organs and tissues BYI 02960-DFA was by far the dominating metabolite accounting for more than 50% of the radioactivity.
The metabolic profiles in urine and faeces were very similar for both sexes but male rats showed a higher rate of metabolite formation as compared to female animals.

The principal metabolic reactions of flupyradifurone in rats were:

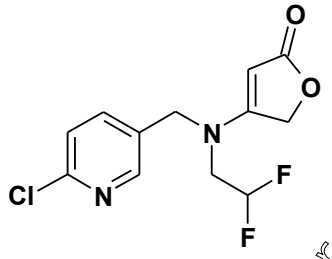
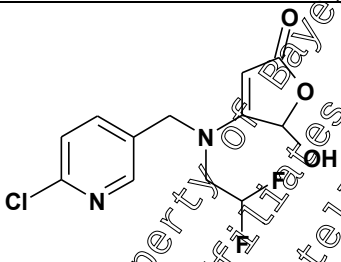
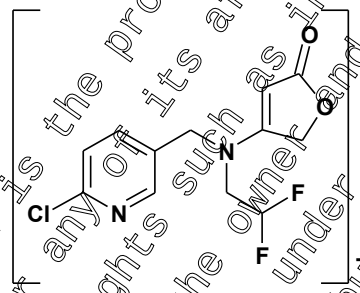
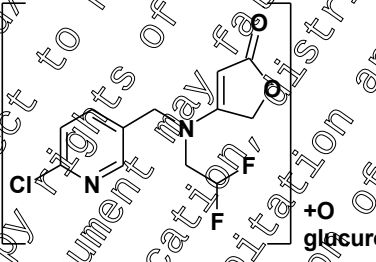
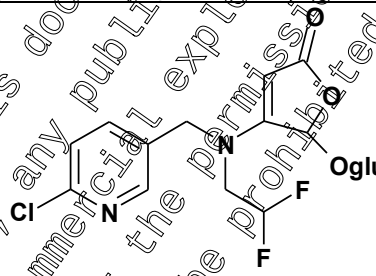
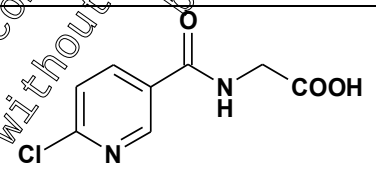
- Hydroxylation followed by conjugation with glucuronic acid or sulfate,
- cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl, and difluoroacetic acid (BYI 02960-DFA),
- cleavage of the molecule at the pyridinylmethylene bridge forming BYI 02960-6-CNA, which was further conjugated with glycine to BYI 02960-hippuric acid and BYI 02960-difluoroethyl-amino-furanone.
- The figure below schematically shows the sites of the molecule which are involved in the metabolic reactions:

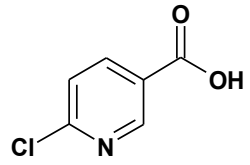
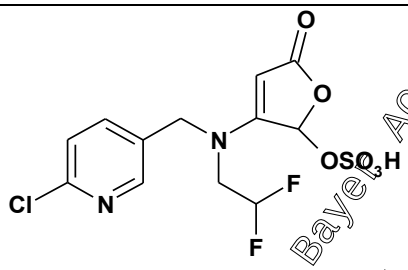
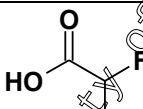
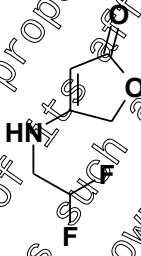
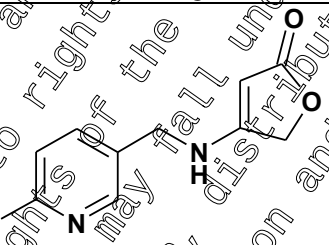


Summarizing the results of the metabolism studies conducted in the rat, a proposed metabolic pathway of BYI 02960 can be described as shown in this figure:



A comprehensive list of metabolites detected in the rat is provided in the following table.

Report Name	Chemical Structure	IUPAC Name
active substance: BYI 02960		4-[[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino]furan-2(5H)-one
BYI 02960-OH		4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-5-hydroxyfuran-2(5H)-one
BYI 02960-iso-OH		
BYI 02960-OH-gluA (isomer 1)		
BYI 02960-OH-gluA (isomer 3)		3-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-5-oxo-2,5-dihydrofuran-2-yl beta-D-glucopyranosiduronic acid
BYI 02960-hippuric acid		N-[(6-chloropyridin-3-yl)carbonyl]glycine

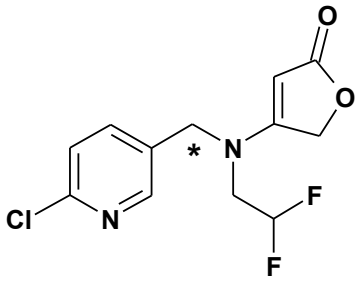
Report Name	Chemical Structure	IUPAC Name
BYI 02960-6-CNA		6-chloronicotinic acid
BYI 02960-OH-SA		3-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-5-oxo-2,5-dihydrofuran-2-yl hydrogen sulfate
BYI 02960-DFA		difluoroacetic acid [free acid]
BYI 02960-difluoroethyl-amino-furanone		4-[(2,2-difluoroethyl)amino]furan-2(5H)-one
BYI 02960-desdifluoroethyl		4-[(6-chloropyridin-3-ylmethyl)amino]furan-2(5H)-one

KIIA 5.1 - Absorption, distribution, excretion and metabolism in mammals

Two studies are summarized in this chapter. The first study report (No. MEF-11/747) describes the absorption, distribution, metabolism, and excretion of the pyridinylmethyl-^{14}C labelled test compound in male and female rats which were dosed with a single low dose, at a single high dose and an intravenous low dose (males only). The excretion of radioactivity was investigated in urine and feces, the radioactivity concentration was determined in the organs and tissues at sacrifice and the metabolites were identified in the excreta. The toxicokinetic behavior of the total radioactivity was investigated by plasma curve analysis.

The second study (Report No. MEF-11/276) describes the distribution and excretion of the pyridinylmethyl-^{14}C labelled test compound in male and female rats after a single oral low dose. In this study, the distribution at different time points was measured by quantitative whole body autoradiography. Excretion was investigated for urine, feces and expired air.

The position of the radiolabel is shown in this figure:



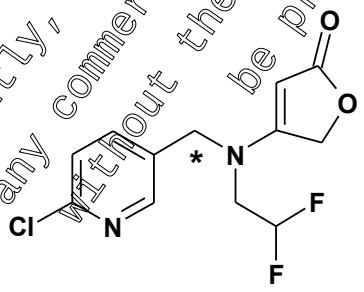
[pyridinylmethyl-^{14}C]BYI 02960

KIIA 5.1.1 - Toxicokinetic studies - Single dose, oral route, in rats

Report:	**KIIA 5.1.1/01, [redacted], 2012**
Title:	[Pyridinylmethyl-^{14}C]BYI 02960 – Absorption, Distribution, Excretion, and Metabolism in the Rat
Report No & Document No	MEF 11/747 M-422210-01-1
Guidelines:	OECD Guideline for Testing Chemicals, 417: Toxicokinetics US EPA Health Effects Test Guidelines OPPTS 870.7485 Metabolism and Pharmacokinetics Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	Yes, fully compliant US EPA - FIFRA Good Laboratory Practice (40 CFR Part 160) Principles of Good Laboratory Practice – German Chemical Law (Chemikaliengesetz), dated 2002-06-20, current version of Annex 1 JAPAN MAFF - Notification on the Good Laboratory Practice Standards for Agricultural Chemicals (11 Nousan 6283) notified 1999-10-01
Testing Facility and Dates	[redacted] Experimental work: 2009-03-24 - 2011-07-12

Executive Summary

The absorption, distribution, excretion, and metabolism of the new insecticide BYI 02960 (common name: flupyradifurone) were investigated in male and female Wistar rats. Groups of four rats were administered orally by gavage with a single dose of BYI 02960 in 0.5% aqueous Tragacanth® at a dose level of 2 mg/kg in the low dose tests and 200 mg/kg in the high dose tests. Additionally, a group of four male rats was administered intravenously with a single dose of BYI 02960 in water at a dose level of 2 mg/kg. The test compound was radiolabelled with ^{14}C in the pyridinylmethyl bridge of the molecule as shown below:



*** denotes the ^{14}C-label position**

The animals were sacrificed 72 h after dosing. Urine and faeces were collected. For each test, the radioactivity levels in plasma were followed by collection of micro samples from each animal over the whole testing period (17 time points). The total radioactivity was determined in excreta and in organs and tissues at sacrifice. Metabolism was investigated in urine and feces.

The total recoveries were almost quantitative since approx. 91 to 103% of the administered dose was found in the excreta and in the body at sacrifice. [Pyridinylmethyl-^{14}C]BYI 02960 was almost completely absorbed after oral administration, which was demonstrated by the bioavailability calculated from the plasma data of low dose tests after oral and i.v. administration, and because of approx. 76 to 90% of the administered radioactivity was detected in the urine and in the body excluding GIT at sacrifice of male and female rats. The absorption commenced immediately after dosing as can be seen from the fast increase of radioactivity concentration in the plasma.

The distribution of the radioactivity within the body was fast and the maximum plasma level (C_{max}) was reached within 1 hour after administration in the low dose tests and within approx. 2 to 4 hours in the high dose tests. From the maximum, the radioactivity level declined slowly to ca. 50% of C_{max} after 4 - 8 hours in the low dose tests and after 8 - 24 hours in the high dose tests. The plasma concentrations showed a further decline to values around the LOQ in the low dose tests and to approx. 0.5% of C_{max} in the high dose tests.

Excretion was fast and mainly renal. Female rats exhibited slightly higher renal excretion rates of approx. 86% and 90% of the administered dose than males with approx. 76% of the dose. The major part of the dose detected in urine (>84%) was excreted within 24 hours after treatment. Excretion was continuing to 72 hours. At the time of sacrifice the radioactive residues in organs and tissues were low and only trace amounts of approx. <0.1 - 0.3% of the given dose was detected in the body and in the GIT. Most of the residues in the organs and tissues of the low dose tests were below 0.01 mg/kg. The highest concentrations of the low dose tests, but still at a low level of ≤0.018 mg/kg were detected in blood cells, the GIT and in the eyes of female rats. The residues of the low and high dose tests were dose-proportional.

Parent compound, three major and five minor metabolites were isolated from urine and four of them identified by spectroscopic methods (LC-MS, 1H-NMR and 2D-1H-NMR). Further identification was obtained by HPLC and TLC co-chromatography and by comparison of the metabolite patterns in HPLC profiles. Identification rates were high and ranged from approx. 83% to 94% of the given dose. A total of further 19 unknown metabolites were characterised by their chromatographic behaviour. All of them were detected in trace amounts of approx. ≤0.1% to 0.9% of the dose. The metabolism results expressed as % of the total dose are summarised in the Table 5.1.1-01.

Table 5.1.1-01: Amounts of metabolites expressed as % of the total dose administered in the excreta of rats after administration of [pyridinylmethyl-^{14}C]BYI 02960

	Male p.o. 2 mg/kg	Female p.o. 2 mg/kg	Male p.o. 200 mg/kg	Female p.o. 200 mg/kg	Male i.v. 2 mg/kg
Parent compound	40.9	77.7	39.6	65.5	47.3
6-CNA	2.4	0.4	6.3	1.3	2.8
Hippuric acid	7.6	1.1	10.5	2.2	5.1
BYI 2960-OH-gluA (isomer 1)	1.8	0.4	1.6	0.7	1.6
BYI 2960-OH-gluA (isomer 3)	2.4	0.4	2.3	1.1	1.0
BYI 2960-des-difluoroethyl	2.2	2.4	1.8	2.7	1.7
BYI 2960-OH-SA	0.2	0.3	0.5	0.5	0.2
BYI 2960-OH	28.9	10.8	24.0	15.1	22.3
BYI 2960-iso-OH	0.4	<0.1	0.4	0.1	0.5
Total identified	86.9	93.7	86.9	89.2	82.5
unknown 1	0.2	0.1	0.3	0.1	0.3
unknown 2	0.7	0.1	1.1	0.3	0.6
unknown 3	0.5	0.1	0.5	0.1	0.4
unknown 4	0.3	0.1	0.5	0.1	0.3
unknown 5	0.3	0.1	0.2	0.1	0.1
unknown 6	0.2	---	0.1	---	---
unknown 7	0.5	0.2	0.2	0.1	0.3
unknown 8	0.2	0.2	0.5	0.3	0.3
unknown 9	0.4	<0.1	0.3	0.1	0.2
unknown 10	0.4	0.1	0.4	0.3	0.2
unknown 11	0.9	0.3	0.8	0.6	0.5
unknown 12	0.2	0.1	0.2	0.2	0.3
unknown 13	0.4	0.3	0.4	0.2	0.3
unknown 14	0.2	0.2	0.3	0.2	0.2
unknown 15	---	0.1	---	0.2	---
unknown 16	0.5	0.1	0.6	0.1	0.5
unknown 17	---	---	---	---	0.1
unknown 18	---	---	<0.1	---	0.1
unknown 19	0.2	0.1	0.2	0.1	0.2
Total characterized	**5.8**	**2.1**	**6.7**	**3.4**	**4.7**
Total	**98.6**	**97.6**	**102.4**	**96.3**	**90.9**

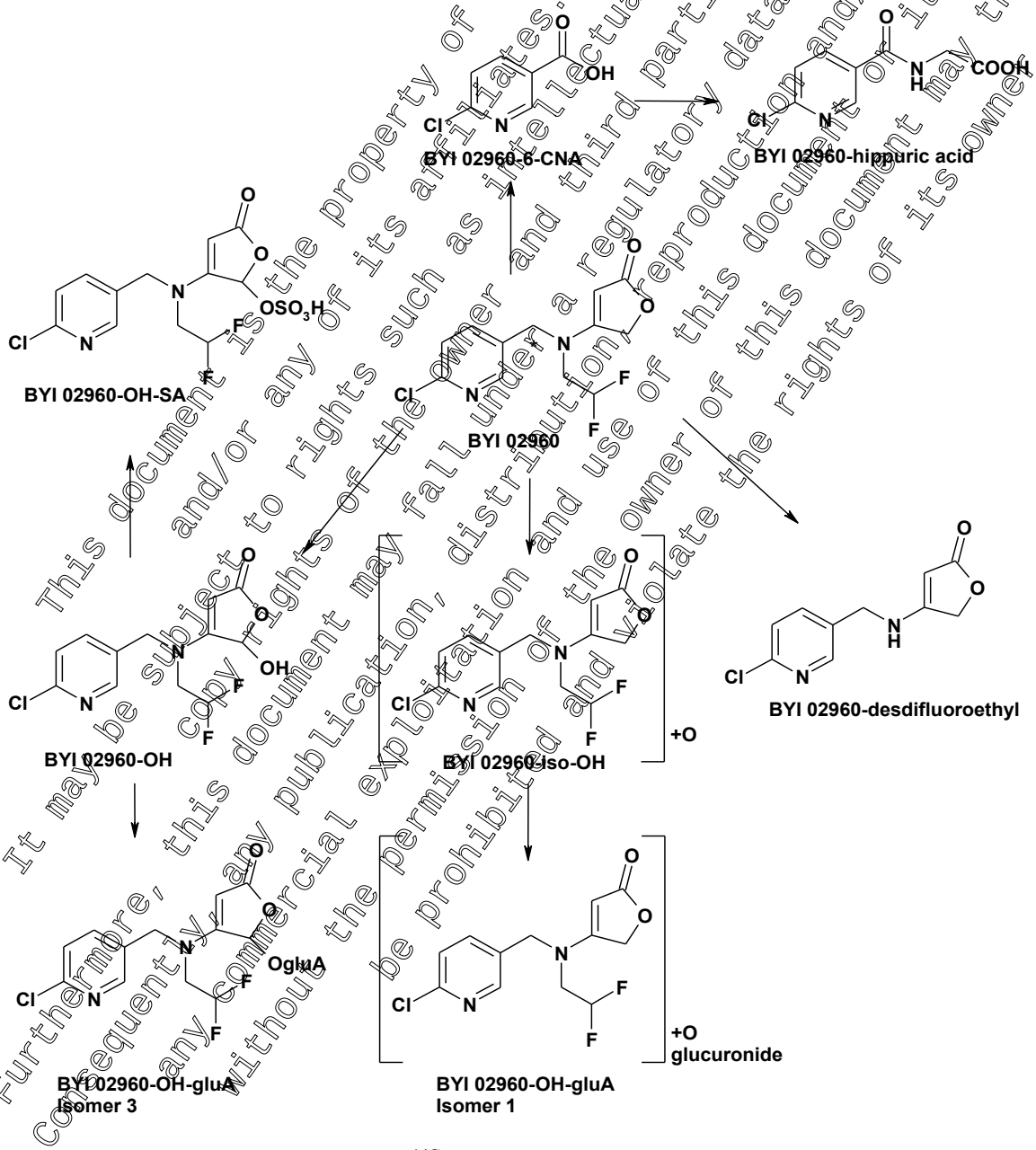
The metabolic profiles in urines and feces were very similar for both sexes and dose rates but male rats exhibited a higher rate of metabolite formation as compared to female rats. The metabolic transformation of BYI 02960 was principally oxidative in nature and took place at least at 3 different structural positions of the molecule.

The principal metabolic reactions of [pyridinylmethyl-^{14}C]BYI 02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid or sulfate,
- cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl, and
- cleavage of the molecule at the pyridinylmethyl bridge forming BYI 02960-6-CNA, which was further conjugated with glycine to BYI 02960-hippuric acid.

The results of the metabolism investigations of the present study are in good accordance with the results of the rat ADME and the organ metabolism studies performed with the furanone-4- and the ethyl-1-label (KIIA 5.1.2/01; KIIA, 5.1.2/03; KIIA 5.1.3/01; KIIA, 5.1.3/02).

The proposed metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in rats is shown below:



*** position of ^{14C} label; gluA = glucuronic acid**

I. Material and Methods

A. Material

1. Test Material:

IUPAC Name:	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name:	BYI 02960
Common name:	Flupyradifurone
Empirical formula:	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass:	288.68 g/mol
Water solubility:	pH 4 = 3.2 g/L, pH 7 = 3.2 g/L pH 9 = 3.0 g/L
Sample ID	BYI 02960-PU-02
Chemical Purity	99.4% (by various methods *e.g.* HPLC, GC)
n-Octanol/water partition coefficient:	pH 4 = 1.2, pH 7 = 1.2, pH 9 = 1.2
Labelling:	[pyridinylmethyl-^{14}C]
Specific radioactivity of the radiolabelled batches:	4.37 MBq/mg = 2.62 x 10^5 dpm/µg = 118.08 µCi/mg = 34.09 Ci/mol
Specific radioactivity used for administration:	4.37 MBq/mg = 2.62 x 10^5 dpm/µg = 118.08 µCi/mg = 34.09 Ci/mol for the low-dose tests; 0.0437 MBq/mg = 2.62 x 10^3 dpm/µg = 1.18 µCi/mg = 0.34 Ci/mol for the high-dose tests
Radiochemical purity:	> 99 % (certified, HPLC and TLC with radiodetection)
Dose level:	2 mg/kg body weight, 200 mg/kg body weight
Vehicle:	0.5 % aqueous tragacanth suspension in oral dose test groups, water in the intravenous test group
Stability of the test material:	The stability of [pyridinylmethyl-^{14}C]BYI 02960 was determined by radio-HPLC of the administration suspensions immediately after dosing

2. Test Animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	Wistar Hsd/Cpb: WU
Breeding facility:	[redacted]
Sex and numbers involved:	12 male animals 8 female animals
Age:	Males: ca. 6-7 weeks at the time of delivery Females: ca. 8-9 weeks at the time of delivery
Body weight:	192 - 242 g at the time of administration
Acclimatization:	Makrolon® cages on wood shavings in the test facility for 7 days prior to the administration.
Identification:	Cage cards on which the study number, test compound name and individual animal number were displayed. Additional labelling with water-insoluble spots on the tail

Housing:	After administration of the radiolabelled test compound individually in Makrolon® metabolism cages under conventional hygienic conditions in air-conditioned rooms Temperature 21 - 25 °C, relative humidity 45 - 71 %. 12/12 hours light / dark cycle, air change 10 - 15 times per hour.
Feed and water:	Rat/mice maintenance long life diet (no. 3883.0.15), supplied by Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland (ca. 16 g per animal and day) Last feeding ca. 16 h prior to dosing. Next feeding ca. 6 h after dosing. Tap water from municipal water supply, *ad libitum*

B. Study Design

1. Dosing

Groups of four rats were administered orally by gavage with a single dose of [pyridinylmethyl-^{14}C]BYI 02960 in 0.5% aqueous Tragacanth® at dose levels of 2 mg/kg bw in the low dose tests and 200 mg/kg bw in the high dose tests after ca. 16 h of fasting. In addition, a group of four male rats was administered intravenously with a single dose of [pyridinylmethyl-^{14}C]BYI 02960 in water at a dose level of 2 mg/kg bw. The dosing suspension (0.21 mg/mL) was prepared in a cold room at 5°C.
The rats of the test groups being orally dosed received the calculated volume by gavage using a syringe attached to an animal-feeding knob cannula. Each animal was dosed with 2 mL of the administration suspension.
The rats of the intravenous test group received the calculated volume (0.5 mL) into the femoral vein. Prior to i.v. administration, the rats were sedated with the analgesic Metamizol® and anesthetised with ether.
The administration volume was based on the nominal average animal weight of 200 g. The concentration of the administration suspensions and solution was calculated to reach an administered amount of about the nominal value of the test compound per kg body weight. Due to different animal weights at administration, the actual doses per kg varied slightly with the body weight. The actual mean administered dose of [pyridinylmethyl-^{14}C]BYI 02960 was 1.97 mg/kg at the oral low dose, 183.6 mg/kg at the oral high dose level and 1.70 mg/kg at the intravenous low dose level. The stability of the test compound in the suspension was assured by radio-HPLC analysis after administration.

2. Collection of excreta

After administration the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine and faeces. Urine was collected separately for each animal in a cryogenic trap cooled with dry ice in intervals of 4 h, 8 h, 12 h, 24 h, 48 h and 72 h. The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction. The radioactivity was determined by LSC.
The faeces samples were collected every 24 h separately for each animal before they were lyophilised, weighed and homogenised. The radioactivity was determined by combustion/LSC.

3. Plasma micro-samples

Blood samples were collected separately for each animal by pressing a capillary coated with heparin in a small cut in the tail vein. The wound was sealed with adhesive tape. The capillaries were centrifuged using a hematocrit centrifuge to separate plasma from the formed blood constituents. After centrifugation, the capillary was broken at the border between plasma and formed constituents and the plasma (approx. 10 - 100 mg) squeezed out onto a small metal dish for weighing. After weighing, this dish was placed into a scintillation vial for radioactivity measurement. Blood samples were collected at 10 min, 20min, 40 min, 1h, 1.5h, 2h, 3h, 4h, 6h, 8h, 24h, 28h, 32h, 48h, 52h, 56h and 72h after dosing from the same animals. Plasma curves could thus be generated for single animals avoiding inter-animal variations. For pharmacokinetic calculations, the average plasma value of the four rats was used.

4. Sacrifice

The rats were sacrificed in Pentobarbital-Na anaesthesia (Narcoren®, supplied by [redacted] Germany) by transection of the cervical vessels and exsanguinated.

5. Plasma, tissues and organs at sacrifice

At sacrifice, blood was collected in heparinised test tubes and separated into plasma and blood cells by centrifugation. After weighing, aliquots from the whole plasma sample were taken for determination of radioactivity by LSC.

Organs and tissues were weighed immediately after dissection and again after lyophilisation. Finally, they were homogenized prior to taking aliquots for the determination of radioactivity by combustion/LSC. For small organs and tissues such as adrenal glands, thyroid, renal fat, only the wet weight was determined before they were solubilised using BTS 450 (Beckman Tissue Solubiliser) and radioassayed by LSC. The radioactivity remaining in the GIT, skin, and residual carcass was determined by combustion/LSC in order to establish the radioactivity balance. The gastro-intestinal tract and an aliquot of depilated skin were lyophilized. After weighing and homogenization, aliquots were taken for determination of radioactivity by combustion/LSC.

6. Measurement of radioactivity

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). All solid samples were combusted in an oxygen atmosphere using an oxidizer. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

7. Toxicokinetic analysis

In this study the software TOPFIT (ver. 2.0) was used to calculate the toxicokinetic parameters by plasma concentration-time curve analysis for the mean equivalent concentration values. A standard 2-compartment disposition model was applied for curve fitting. Compartment models attempt to mathematically describe the processes of absorption, entry into the systemic circulation, distribution to organs or tissues where metabolism can occur, and subsequent excretion.

8. Analytical methods

Samples were analyzed by radio HPLC, radio TLC, LC-MS and NMR methods.

9. High Performance Liquid Chromatography (HPLC)

HPLC-radioactivity analysis was performed on Agilent 1100 or 1200 systems with radiometric- and UV-detection. The separation was carried out on a reversed phase column using an acidic or a neutral water / acetonitrile gradient. Detailed information of the HPLC-methods and instruments set-up can be found in the report. All solvents were of HPLC-quality.

The chromatograms were recorded and quantitatively evaluated using the software package GINA (raytest, Benzstraße 4, D-75334 Straubenhardt, Germany). The ^{14}C-trace of a chromatogram was divided into regions of interest (ROI's), according to the separated radioactive peaks. Additionally, background regions (BKG's) were defined and used for background correction. The background corrected area counts from all regions of interest were used for calculation of the quantitative distribution of the individual components in the sample. A quantifiable radioactive peak was considered relevant if the signal was approx. 2.5 times above the background noise.

For co-chromatography in HPLC, the sample was mixed with the reference compound before injection. The detection was achieved either by UV-absorption of the non-radiolabelled or by ^{14}C-detection of the radiolabelled reference compound. Chromatographic matching with the non-radiolabelled reference compound was assessed by comparison of the UV-trace and the associated ^{14}C-trace. Chromatographic matching with the radiolabelled reference compound was assessed by comparison of the ^{14}C-chromatogram of the mixture with the ^{14}C-chromatogram of the sample without the reference compound.

10. Thin Layer Chromatography (TLC)

Thin layer co-chromatography (silica 60 F_{254}, normal phase) with the non-radiolabelled reference compound was used to detect and identify BYI 02960-OH that had been isolated from urine. The samples were spotted on the TLC-plates using a Linomat IV - instrument (Camag, Berlin, Germany). The plates were developed using dichloromethane / methanol / ammonium hydroxide solution 25% (85 / 13 / 2; v/v/v) as solvent a system.

The TLC-bands or spots were visualized under UV-light (254 nm). The radioactive zones were detected using a Fujibas® 2000 bio imaging system (Fuji, Japan & raytest, Straubenhard, Germany). Fujibas® imaging plates were exposed to the TLC-plates. The imaging data were identified with Basreader software (version 2.13e, raytest, Straubenhard, Germany. Evaluation and visualization of recorded data was performed with AIDA software (raytest, Straubenhard, Germany). The detection limit (LOD) was 5 - 10 dpm/zone for ^{14}C after an exposure of at least 14 hours.

For TLC co-chromatography, the solutions of the reference compound and the sample were applied individually to the plate each as an approx. 1.5 - 2 cm-wide band. Both solutions were also applied side by side as approx. 4 cm-wide bands with an overlapping part of approx. 2 cm in the middle. After development of the TLC plate, chromatographic correspondence with the reference compound was assessed by analysis of the individual radioluminogram.

11. Mass spectroscopy

Electro-spray ionization MS spectra (ESI) were obtained either with a TSQ 7000 instrument or with a LTQ Orbitrap XL mass spectrometer (Thermo or Finnigan, respectively, San Jose, CA, U.S.A.). The HPLC instrument used for chromatography was an Agilent HP1100 (Agilent, Waldbronn, Germany). The eluate of the HPLC-column was split between the UV-detector followed by a radioactivity detector (Ramona Star, Raytest, Straubenhardt, Germany) and the MS spectrometer.

12. Nuclear Magnetic Resonance spectroscopy

The 600 MHz 1H-NMR spectra were recorded using a BRUKER AV 600 instrument (Bruker, Karlsruhe, Germany).

13. Preparation of samples, extraction and analysis

Urine samples collected from 0 to 48 h were combined to representative pools of each test group and used for metabolic profiling without further sample preparation. The amount of radioactivity applied for analysis was calculated from the in-life data of the respective samples. The faeces samples of the first sampling interval (0-24 h) from all four animals of a test group were combined. The level of radioactivity in the pool sample was calculated from the in-life date of the respective samples. These pool samples were extracted with acetonitrile/water mixtures. The extracts containing the majority of the radioactive residue were combined, further purified by SPE on a C18 cartridge and then concentrated for subsequent HPLC analysis. The radioactivity in the post extraction solids was determined by LSC following combustion analysis.

14. Identification / Characterisation and Quantification of Residues

All urine samples and faeces extracts were analysed by HPLC using the profiling method BYI02960_ADME based on a reversed phase column with a buffer pH 7/ACN gradient and UV- and radio-detection.

Parent compound and metabolites were identified by spectroscopic evidence (LC-MS and high resolution LC-MS analysis, and 1H-NMR and 2D-1H-NMR analysis) following isolation and purification from the urine pool of the high dose test with females.

Further identification was achieved by HPLC and TLC co-chromatography of urine samples or isolated peaks with the isolated and identified metabolites and with non-radiolabelled reference compounds, by HPLC co-chromatography with the egg extract and the isolated metabolite (BYI 02960-OH-SA) from this egg extract of the laying hen metabolism study performed with [pyridinylmethyl-14C]BYI 02960 (Reference).

Identification of glucuronide conjugates was achieved by enzyme treatment with glucuronidase / arylsulfatase together with comparison of the HPLC profiles of the metabolites before and after cleavage to the profiles of urine, faeces extract and reference compounds.

Comparison of urine profiles of the present study with the corresponding ones of the rat organ metabolism study (KIIA 5.1.2/03) and with urine of the rat ADME study (KIIA 5.1.2/01) both performed with [furanone-4-14C]BYI 02960 also proved to be helpful for identification.

Further details on the methods used for identification, characterisation and quantification are provided in the report.

II. Results and Discussion

A. Recovery

The total recovery for the orally administered tests was almost quantitative since between approx. 97 and 103% of the administered dose was found in the excreta and the body of male and females rats at sacrifice. The total recovery was slightly lower and accounted for approx. 91% of the administered dose in the low dose test with intravenous administration of male rats. The results in percent of the given dose in urine, faeces, organs and tissues at sacrifice are shown in Table 5.1.1-02.

Table 5.1.1-02: Recovery of radioactivity in urine, gastrointestinal tract and the body following oral or intravenous dosing of [pyridinylmethyl-^{14}C] BYI 02960. Data are presented as % of the administered radioactivity

Dose [mg/kg bw]	Male 2 p.o.	Female 2 p.o.	Male 200 p.o.	Female 200 p.o.	Male 2 i.v.
Faeces	23.09	7.49	26.14	10.32	14.64
Urine	75.45	90.07	76.26	85.95	76.24
Sum of excreta	**98.55**	**97.56**	**102.40**	**96.26**	**90.88**
Body without GIT	0.119	0.064	0.128	0.241	0.141
GIT	0.069	0.010	0.086	0.064	0.086
Total in body	**0.188**	**0.074**	**0.214**	**0.306**	**0.227**
Balance	**98.73**	**97.63**	**102.60**	**96.57**	**91.11**

B. Absorption

[Pyridinylmethyl-^{14}C]BYI 02960 was almost completely absorbed in male and female rats. This was demonstrated by the high bioavailability factor evaluated from the comparison of the AUC obtained in low dose tests with male rats after oral and intravenous administration and by the radioactivity detected in the urine and in the body without GIT. The amounts of radioactivity in the urine and in the body without GIT was identical in the low and high dose tests with male rats and represented approx. 76% of the dose. The corresponding values for females were even higher and amounted to approx. 86 and 90% of the dose (see Table 5.1.1-02). The absorption commenced immediately after dosing as can be seen from the quick increase of radioactivity in plasma micro samples (see Table 5.1.1-03).

Table 5.1.1-03: Time course of radioactivity in the plasma following oral or intravenous dosing of [pyridinylmethyl-^{14}C]-BYI 02960

Dose [mg/kg]	Concentration [mg a.s. equiv./kg]				
	Male 2 p.o.	Female 2 p.o.	Male 200 p.o.	Female 200 p.o.	Male 2 i.v.
0.17	0.4465	0.5287	32.5100	30.9100	1.6070
0.33	1.0380	1.2010	66.3400	57.2100	1.6970
0.67	1.4690	1.7240	88.9400	74.9300	1.7670
1	1.7130	1.8540	94.3200	84.4500	1.7060
1.5	1.6740	1.7920	96.0300	87.7900	1.5570
2	1.5500	1.7630	96.8600	92.7700	1.3850
3	1.3410	1.6100	95.6200	98.3200	1.1180
4	1.1990	1.4500	96.3100	99.9900	0.8976
6	0.7990	1.1350	80.6500	91.7700	0.6250
8	0.5281	0.8420	69.2600	81.9800	0.4357
24	0.0200	0.0354	6.4460	22.4000	0.0193
28	0.0134	0.0228	3.3800	17.8300	0.0157
32	0.0100	0.0157	2.1660	10.1800	0.0103
48	---	0.0052	0.6626	1.4800	0.0062
52	---	---	0.6165	1.2700	0.0053
46	---	---	0.5762	0.9593	0.0038
72	---	---	0.3602	0.5697	0.0044

C. Distribution and plasma kinetics

The test compound was quickly absorbed and distributed in the blood as can be seen from the analysis of plasma at different time points in Table 5.1.1-03.
The maximum level of radioactivity (C_{max}) was already reached 1 hour after administration in all low dose tests. At this time, the radioactivity level in plasma corresponded approximately to the equidistribution concentration for female rats (C_{norm} = 0.9373), to ca. 88% of the equidistribution concentration for male rats after oral administration and to ca. 101% for male rats after i.v. administration (see Table 5.1.1.03). The maximum level of radioactivity (C_{max}) in the high dose tests with male and female rats was reached at approx. 2 hours after administration for males and at approx. 4 hours after administration for females.
The plasma concentrations declined to approx. 50% of C_{max} within 4 - 8 hours and to approx. 1 - 2% of C_{max} within 24 hours in the low tests with male and female rats. In the high dose tests with male and female rats, the decline phases were slightly delayed, the level of approx. 50% of C_{max} was reached within 8 to 24 hours and approx. 1 - 2% at 48 hours. The concentrations steadily declined further. At the time of sacrifice, 72 hours after administration, the plasma concentrations were below or around the LOQ for male and female animals of the low dose tests and at approx. 0.5% of C_{max} in the high dose tests.
The mean values of the dose normalised plasma concentrations from the 4 animals of each test were used for a biokinetic modelling using the TOPFIT software. The evaluation was performed with dose normalised data to allow for better comparison of the low dose tests amongst themselves and to the high dose tests. About 32 - 48 hours post administration, the concentrations in plasma of the low dose tests were below or close to the LOQ. Hence, biokinetic modelling of the low dose tests was performed for the time range of 0 to 32 hours post administration. The biokinetic modelling of the high dose tests was performed for the same time range for direct comparison with the low dose tests. Good fits of the measured and calculated values were achieved in all tests with a two compartment model.
The plasma curves of male rats of the low dose tests after oral and i.v. administration were quite comparable (Figure 5.1.1-01). The plasma curve of the low dose females was slightly higher than the male ones but with a similar curve shape. The plasma curves of the high dose tests showed a broader maximum for males and a distinctly broader maximum for females when compared with the ones of the low dose tests due to an obviously delayed absorption of the test compound from the gastrointestinal tract.
The biokinetic parameters were comparable in the three low dose tests. Only the AUC value for females of the low dose test was approx. 1.3 times higher than for males, which is related to the slightly broader peak maximum of the plasma curve of female rats. In the high dose tests, the AUC value for males was proportional to the dose. For females, the AUC value of the high dose test was approx. 1.2 times higher than for the low dose test with females and approx. 1.6 times higher than for the high dose test with males. According to the broader maximum of the plasma curve of female rats in the high dose test, C_{max} was reached later than in males (approx. 4 hours instead of 2 hours).
The biokinetic model confirmed a fast absorption phase in all tests with short half-lives of max. 0.2 hours followed by a short elimination phase with half-lives of about 3 - 4 hours. Only females in the high dose test showed a slightly longer elimination phase with a half-life of about 8 hours.
The mean residence times in the low dose tests were in the range of approx. 6 - 7 hours. The mean residence times were slightly longer in the high dose tests and amounted to about 9 hours for males and

13 hours for females. An overview of all important results of the TOPFIT analysis is presented in Table 5.1.1-04.

Figure 5.1.1-01: Comparison of dose normalised plasma curves following oral administration of low and high doses of [pyridinylmethyl-^{14}C]BYI 02960 to male and female rats

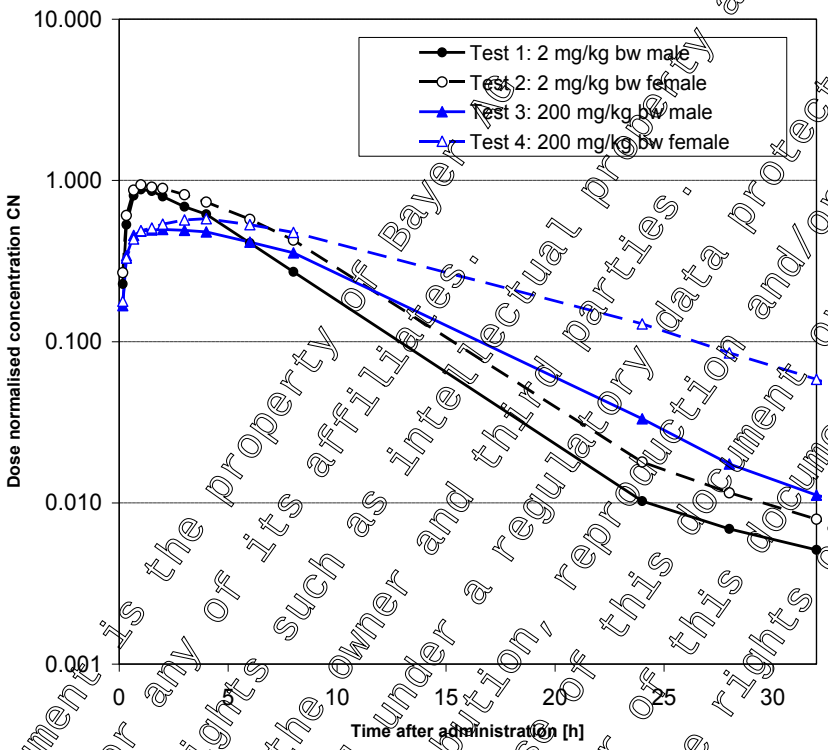


Table 5.1.1-04: Pharmacokinetic parameters and calculation of the bioavailability of [pyridinylmethyl-^{14}C]BYI 02960 after oral administration to male and female rats, and after i.v. administration to male rats

Dose (mg/kg bw)	Male 2 p.o.	Female 2 p.o.	Male 200 p.o.	Female 200 p.o.	Male 2 i.v.
t_{max} [h] measured	1.0	1.0	2.0	4.0	0.67
t_{max} [h] calculated	1.13	1.15	2.23	3.35	0.38
C_{max} measured values	0.878	0.937	0.497	0.578	1.04
C_{max} calculated (fitted) values	0.880	0.929	0.500	0.582	1.0400
$t_{1/2\ abs}$ [h]	0.21	0.17	0.13	0.17	0.06
$t_{1/2\ elim\ 1}$ [h]	3.9	3.0	3.6	8.1	3.8
$AUC_{0-\infty}$ [mg/L*h]	6.10	7.96	6.16	9.73	6.55
MRT_{tot} [h]	6.07	6.69	8.70	13.10	5.71

C. Excretion

The major route of excretion in all tests was renal. In total, approx. 76% of the administered dose was detected in the urine of male rats. Renal excretion was slightly higher for females where between

approx. 86 and 90% of the administered dose was renally excreted. The majority of the radioactivity (> approx. 84%) detected in urine was excreted within 24 hours.

Faecal excretion accounted for ca. 7 - 10% of the given dose in females and was slightly higher for males, where between approx. 15 and 26% was excreted with the feces. Also the major part of faecal residues, approx. 89 - 92% of the radioactivity in faeces in the low dose tests and approx. 47 and 77% in the high dose tests, was excreted within the first day after treatment and was almost complete 48 hours post administration.

These results are in good accordance to the excretion behavior detected in all other rat studies. The cumulative excretion results are summarised in Table 5.1.1-05.

Table 5.1.1-05: Cumulative excretion of radioactive residues via urine and faeces after oral administration of [pyridinylmethyl-^{14}C]BYI 02960 to male and female rats, and after i.v. administration to male rats

Dose [mg/kg bw]	Male 2 p.o.	Female 2 p.o.	Male 200 p.o.	Female 200 p.o.	Male 2 i.v.
Feces					
24	20.55	6.86	20.22	4.90	13.31
48	22.82	7.38	25.84	9.64	14.41
72	23.09	7.49	26.14	10.32	14.64
Urine					
4	17.25	5.34	14.89	9.50	26.12
8	40.99	33.80	31.33	28.56	44.02
12	51.67	---	---	---	---
24	72.61	86.95	68.26	72.15	73.80
48	75.15	89.77	75.56	84.15	75.75
72	75.45	90.07	76.26	85.95	76.24
Total excreted	**98.55**	**97.56**	**102.40**	**96.26**	**90.88**

D. Radioactive residues in organs and tissues at sacrifice

Approximately 0.1 - 0.3% of the total dose was detected in the body of male and female rats at the time of sacrifice 72 hours after oral and i.v. administration; only a trace amount of 0.01 - 0.09% was found in the GIT.

Residual concentrations of radioactivity were low in the low dose tests and ranged from 0.0007 to 0.0175 mg/kg. The highest concentrations were detected in blood cells and the GIT, and in the eyes of female rats. But basically, levels for most organs and tissues were very similar and in the range of approx. 0.001 to 0.007 mg/kg. The residual concentrations of the high dose tests were almost dose proportional and ranged from 0.0859 to 2.345 mg/kg. The highest concentrations were also detected in blood cells and the GIT, as well as in the eyes of female rats. For most organs and tissues the residue concentrations in males were slightly higher as compared to females.

The equivalent concentrations of the residual radioactivity in organs and tissues at sacrifice are presented in Table 5.1.1-06.

Table 5.1.1-06: Total radioactive residues in organs and tissues at sacrifice after administration of [pyridinylmethyl-^{14}C]BYI 02960 to male and female rats. Data are presented as equivalent concentration [mg a.s. equiv./kg]

Sex Dose [mg/kg bw]	Male 2 p.o.	Female 2 p.o.	Male 200 p.o.	Female 200 p.o.	Male 2 i.v.
Blood cells	0.0175	0.0067	2.3450	1.5770	0.0158
Plasma	0.0020	0.0013	0.2989	0.2963	0.0025
Carcass	0.0021	0.0011	0.1794	0.2377	0.0020
Heart	0.0024	n.c.	0.2643	0.3328	0.0023
Brain	0.0008	n.c.	0.0859	0.0922	0.0008
Kidneys	0.0064	0.0033	0.7925	0.6691	0.0067
Liver	0.0068	0.0054	0.8741	0.7720	0.0063
GIT	0.0141	0.0019	1.7290	1.1450	0.0167
Testes	0.0008	---	0.1020	---	0.0011
Ovaries	---	n.c.	---	0.2880	---
Uterus	---	0.0016	---	0.4599	---
Adrenal gland	0.0048	0.0032	0.4436	0.5606	0.0045
Harderian gland	0.0050	0.0022	0.4101	0.7218	0.0034
Thyroid	n.c.	n.c.	n.c.	n.c.	n.c.
Spleen	0.0030	0.0017	0.3586	0.3398	0.0032
Lung	0.0060	0.0035	0.6648	0.5665	0.0054
Eyes	0.0064	0.0133	0.5996	1.3430	0.0066
Skin	0.0018	0.0014	0.2354	0.9710	0.0024
Bone (femur)	n.c.	0.0023	0.1771	0.2794	0.0024
Fat (perirenal)	0.0018	n.c.	n.c.	0.1245	0.0030
Muscle (leg)	0.0008	0.0007	0.1206	0.2098	0.0012

n.c. : not calculated

E. Identification / characterization and quantification of residues

The following strategy was used for identification of parent compound and metabolites:
The metabolic profiles of urine pools and faeces extracts of the different test groups were compared with each other. Subsequently, the profiles of faeces extracts and urine pool samples were also compared in order to assign the parent compound and metabolites in these fractions.
The urine of the high dose test with female rats was subjected to a Chem elut clean up and the obtained extracts were then fractionated by HPLC. The isolated parent compound and the metabolites were purified by HPLC and identified with spectroscopic methods (LC-MS, high resolution LC-MS and ^{1}H-NMR and 2D-^{1}H-NMR). Parent compound and metabolites were identified in urine by HPLC co-chromatography with available non-radiolabelled reference compounds.
BYI 2960-OH-SA was identified by comparison of the metabolic pattern in urine with the pattern of egg extract from a laying hen study (Reference) and by HPLC chromatography of urine with BYI 02960-OH-SA isolated from the egg extract.
The two isomers of BYI 02960-OH-gluA (isomer 1 and 3) were identified by comparison of the metabolic pattern of urine from the present study with urine samples from the rat ADME study (KIIA 5.1.2/01) and with urine from the organ metabolism study (KIIA 5.1.2/03), both performed with the

furanone-4-^{14}C label. In this organ metabolism study, all major compounds in urine had been identified by LC-MS/MS and/or HPLC, TLC co-chromatography or comparison. The corresponding hydroxyl aglyca of these metabolites were assigned and identified by HPLC co-chromatography and comparison of HPLC profiles after enzymatic cleavage.
Parent compound and metabolites were quantified in urine samples and in faeces extracts by integration of the ^{14}C-signals in the HPLC-chromatograms. The stability of the metabolic profiles in urine samples and faeces extracts was demonstrated by re-analysis of representative samples with the HPLC profiling method BYI02960_ADME after storage of about 20 months for urine samples and about 14 months for faeces extracts in a freezer.

F. Metabolites in urine

The main compound in urine was the unchanged parent compound representing between approx. 36% and 44% of the total dose administered in male rats. The amount of the parent compound in the urine of female rats was higher and accounted for approx. 74% of the administered dose in the low dose and for approx. 61% in the high dose test.
Eight metabolites were detected in urine, BYI 02960-OH was the major metabolite in the urine of male and female rats accounting for ca. 9% - 18% of the dose. Two further metabolites were prominent in the urine of male rats, BYI 02960-hippuric acid accounting for ca. 5% - 10% of the administered dose and BYI 02960-6-CNA accounting for ca. 2% - 6% of the dose. These two metabolites were also detected in the urine of female rats but at lower amounts of only ca. <1% - 2% of the administered dose. Other identified metabolites were BYI 02960-des-difluoroethyl, BYI 02960-OH-glcA (isomer 1 and 3), BYI 02960-OH-SA and BYI 02960-iso-OH, none of them accounting for more than 3% of the dose.
In total, ca. 76% of the total dose was detected in urine of male rats and approx. 84% to 90% in the urine of female rats. Identified metabolites in urine represented approx. 71% of the administered dose in male rats and approx. 81% and 88% in female rats.
A total of further 19 unknown metabolites were characterised by their HPLC behaviour. Each of them accounted for ≤1% of the administered dose and most of them occurred at trace levels of only 0.1 and 0.2% of the dose. The characterised metabolites represented in total approx. 2% to 5% of the dose.
The results are summarized in Table 5.1.1-07. The peak numbers in the table correlate with those in the representative chromatogram of the urine (12 - 24 h) of the low dosed male rats (Figure 5.1.1-02).

Figure 5.1.1.02: Metabolic profile of the urine of male rats orally dosed with 2 mg/kg [pyridinylmethyl-^{14}C]BYI 02960, sampling interval 12 - 24h

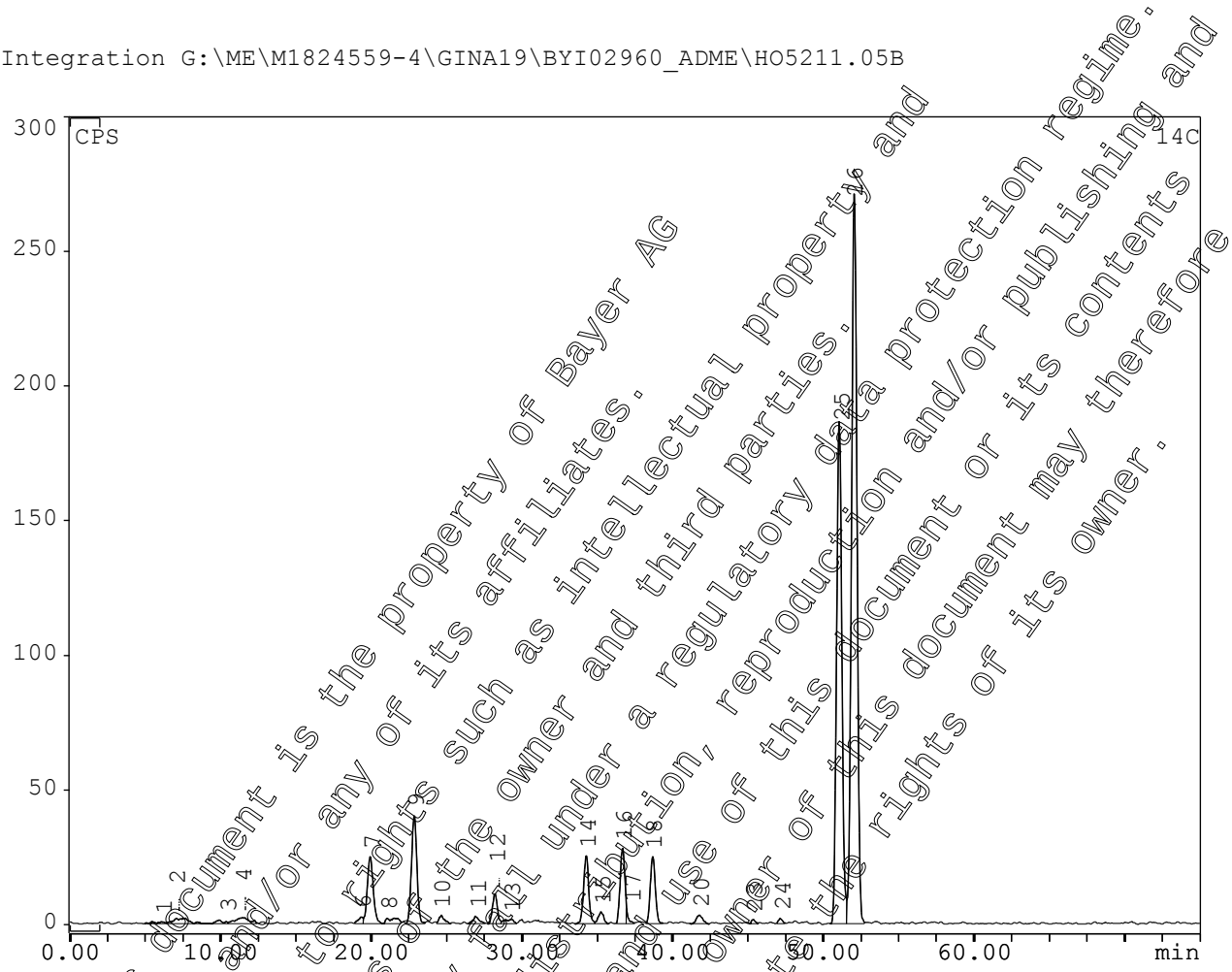


Table 5.1.1-07: Identified metabolites in urine and faeces after administration of [pyridinylmethyl-^{14}C]BYI 02960 to male and female rats. Data are presented as % of the given dose

Peak no.		Male, p.o. 2 mg/kg	Female, p.o. 2 mg/kg	Male, p.o. 200 mg/kg	Female, p.o. 200 mg/kg	Male, i.v. 2 mg/kg
26	Parent compound	40.9	77.7	39.6	65.5	47.3
7	BYI 2960-6-CNA	2.4	0.4	6.3	1.3	2.8
9	Hippuric acid	7.6	1.1	10.5	2.2	5.1
14	BYI 2960-OH-gluA (isomer 1)	1.8	0.4	1.6	0.7	1.6
16	BYI 2960-OH-gluA (isomer 3)	2.4	0.4	2.3	1.1	1.0
18	BYI 2960-des-difluoroethyl	2.2	2.4	1.8	2.7	1.7
24	BYI 2960-OH-SA	0.2	0.3	0.5	0.5	0.2
25	BYI 2960-OH	28.9	10.8	24.0	15.1	22.2
27	BYI 2960-iso-OH	0.4	<0.1	0.4	0.1	0.5
	Total identified	**86.9**	**93.7**	**86.9**	**89.2**	**82.5**
1	unknown 1	0.2	0.1	0.3	0.1	0.3
2	unknown 2	0.7	0.1	1.1	0.3	0.6
3	unknown 3	0.5	0.1	0.5	0.1	0.4
4	unknown 4	0.3	0.1	0.5	0.3	0.3
5	unknown 5	0.3	0.1	0.2	0.1	0.1
5a	unknown 6	0.2	---	0.1	---	---
6	unknown 7	0.5	0.2	0.2	0.1	0.3
8	unknown 8	0.4	0.2	0.5	0.3	0.3
10	unknown 9	0.4	0.1	0.3	0.1	0.2
11	unknown 10	0.1	0.1	0.4	0.3	0.2
12	unknown 11	0.9	0.3	0.8	0.6	0.5
13	unknown 12	0.2	0.1	0.2	0.2	0.3
15	unknown 13	0.4	0.3	0.4	0.2	0.3
17	unknown 14	0.2	0.2	0.3	0.2	0.2
19	unknown 15	---	0.1	---	0.2	---
20	unknown 16	0.5	0.1	0.6	0.1	0.5
21	unknown 17	---	---	---	---	0.1
22	unknown 18	---	---	<0.1	---	0.1
23	unknown 19	0.2	0.1	0.2	0.1	0.2
	Total characterized	**5.8**	**2.1**	**6.7**	**3.4**	**4.7**
	Total	**92.7**	**95.9**	**93.7**	**92.7**	**87.2**

G. Metabolites in feces

The unchanged parent compound in faeces represented approx. 3% to 4% of the total dose. Another main constituent of the faecal residues was metabolite BYI 02960-OH which accounted for approx. 6% to 11% of the dose in male rats and for slightly lower amounts of approx. 2 - 3% in female rats. All other metabolites identified in urine, except BYI 02960-OH-gluA, isomer 1, were also detected in faeces but only at trace amounts of ≤0.4% of the dose.

In total, approx. 13% - 23% of the dose was detected in faeces of male rats and approx. 7% and 10% in females of which the major part (approx. 6% - 16% of the dose) was identified.
In total 16 unknown metabolites were characterised by their HPLC behaviour. None of them occurred at levels above 0.2% of the administered dose. The characterised metabolites represented in total approx. <1% to 2% of the dose. The results are listed in Table 5.1.1.07 as the sum of urine and faeces.

H. Comparison of the metabolic profiles

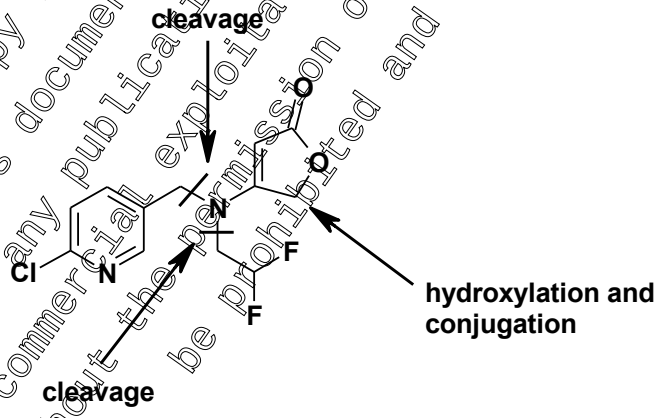
BYI 02960 was metabolised to numerous metabolites, most of them being minor ones. The parent compound was the predominant part of the radioactivity in urine of male and female rats. In faeces samples of male rats, the metabolite BYI 02960-OH was more prominent than the parent compound. Two metabolites, BYI 02960-6-CNA and BYI 02960-hippuric acid were also prominent in male rats but not in females. All other identified and characterised metabolites represented a minor part of the dose.
The metabolic profiles in urine and faeces were very similar for both sexes but male rats showed a higher rate of metabolite formation as compared to female animals.

I. Biotransformation pathway
The principal metabolic reactions of [pyridinylmethyl-^{14}C]BYI02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid or sulfate,
- cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl and
- cleavage of the molecule at the pyridinylmethyl bridge forming BYI 02960-6-CNA, which was further conjugated with glycine to BYI 02960-hippuric acid.

Figure 5.1.1-03 schematically shows the positions in the molecule, which are involved in the metabolic reactions: The proposed biotransformation pathway of [pyridinylmethyl-^{14}C] BYI 02960 is presented in Figure 5.1.1-04.

Figure 5.1.1-03: Positions involved in metabolic degradation of BYI 02960

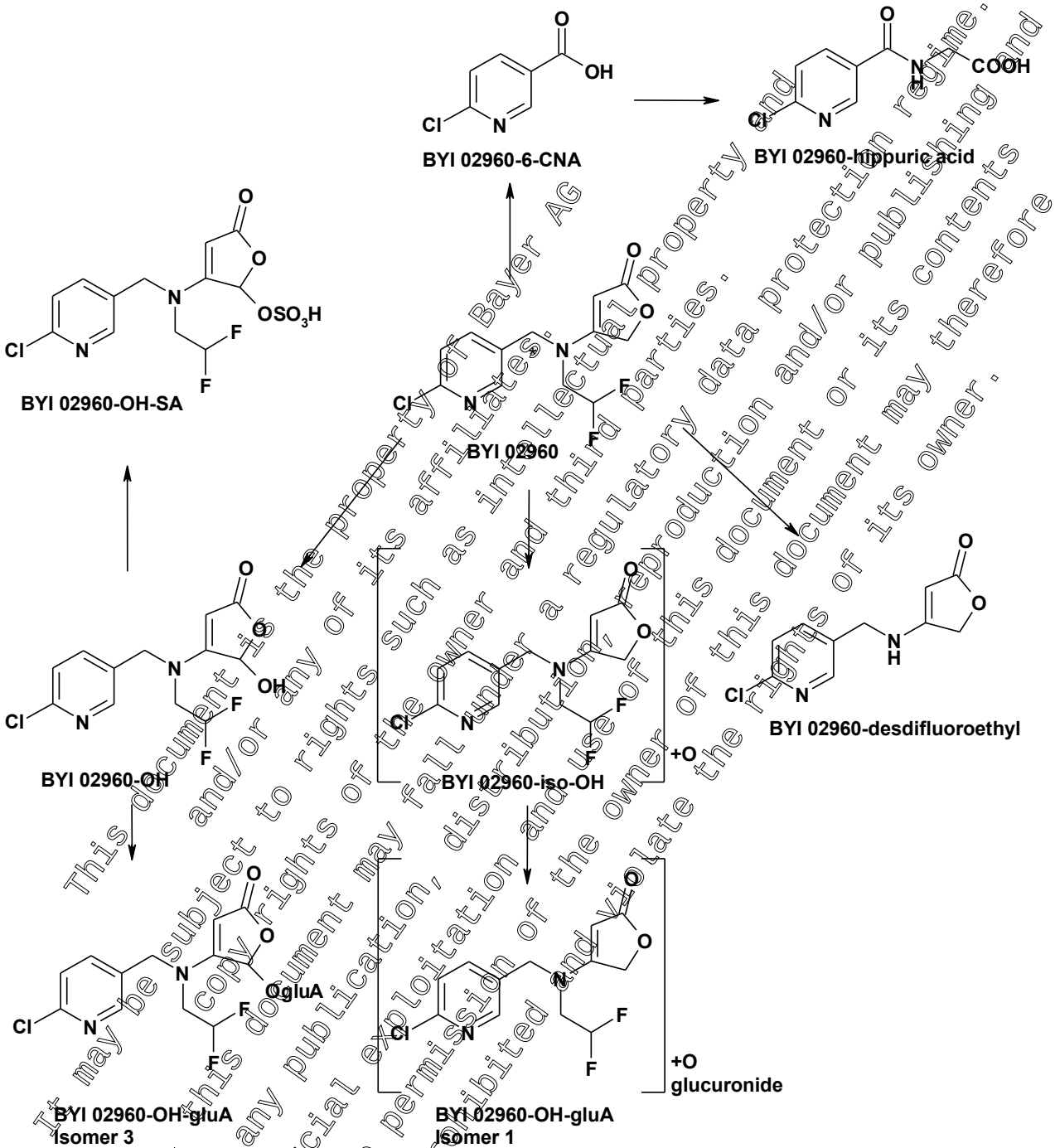


III. Conclusions

The kinetic and metabolic behaviour of [pyridinylmethyl-^{14}C]BYI 02960 in male and female Wistar rats after low and high oral and intravenous dosage can be characterised by the following observations:

- The test compound was completely absorbed in all tests as demonstrated by the high bioavailability after oral administration and by the high amounts of radioactivity detected in urine and the body without GIT at sacrifice.
- The distribution of the radioactivity in the body was fast and the maximum plasma level (C_{max}) was reached within one hour after administration in the low dose tests and within approx. 2 to 4 hours in the high dose tests. From the maximum, the radioactivity level declined to ca. 50% of C_{max} after 4 - 8 hours in the low dose tests and after 8 - 24 hours in the high dose tests. The plasma concentrations showed a further decline to the LOQ in the low dose tests and to approx. 0.5% of C_{max} in the high dose tests.
- Excretion of radioactivity was fast and mainly renal. Female rats showed slightly higher renal excretion rates of approx. 86% and 90% of the administered dose than males with approx. 76% of the dose.
- The major part of the dose (>84%) was excreted within 24 hours after treatment. But excretion was continuing until sacrifice.
- At sacrifice, the residues were low and only trace amounts of approx. <0.1 - 0.3% of the dose administered was detected in the body and in the GIT. Most of the residues of the low dose tests were below 0.01 mg/kg. The highest concentrations, but also at a low level of ≤0.018 mg/kg was detected in blood cells, in the GIT of male rats and in the eyes of female rats. The residues of the low and high dose tests were almost dose proportional.
- Parent compound, three major and five minor metabolites were identified in all samples. Identification rates were high and amounted to approx. 83% - 94% of the given dose.
- A further 19 unknown metabolites were characterised by their chromatographic behaviour. All of them were detected at trace amounts of approx. <0.1% to 0.9% of the dose.
- The metabolic transformation of BYI 02960 was principally oxidative in nature and took place at least at 3 different structural positions.
- The results of the present study are in good accordance with those obtained in the other rat studies with BYI 02960 labelled in different positions.

Figure 5.1.1-04: Proposed metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in rats



* position of ^{14}C label; gluA = glucuronic acid

Report:	**KIIA 5.1.1/02, [redacted], J; [redacted]; 2011**
Title:	Quantitative whole body autoradiography of [pyridinylmethyl-^{14}C]BYI 02960 in male and female rats: distribution of total radioactivity and elimination from blood, organs and tissues after single oral administration including determination of radioactivity in the excreta and exhaled $^{14}CO_2$
Report No & Document No	MEF 11/276 M-409993-01-2
Guidelines:	OECD Guideline for Testing Chemicals, 417: Toxicokinetics US EPA Health Effects Test Guidelines OPPTS 870.7485 Metabolism and Pharmacokinetics PMRA Regulatory Directive Dir2005-01: Section 11: Toxicokinetic studies Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	Yes, fully compliant US EPA – FIFRA Good Laboratory Practice (40 CFR Part 160) Principles of Good Laboratory Practice – German Chemical Law (Chemikalengesetz), dated 2002-06-20, current version of Annex 1 JAPAN MAFF - Notification on the Good Laboratory Practice Standards for Agricultural Chemicals (11 Nousan 6283) notified 1999-10-01
Testing Facility and Dates	[redacted] Experimental work: 2009-03-05 - 2009-09-15

Executive Summary

BYI 02960 was readily absorbed from the gastrointestinal tract and distributed throughout the body immediately after administration. The excretion of radioactivity via urine and faeces was almost complete after 2 days with renal excretion as the predominant route. For male rats, ca. 72 to 85% of the administered dose was recovered in urine and 21 to 27% in faeces. Whereas for the female rats the urinary excretion was slightly higher with ca. 89 - 93%, less than 0.1% of the administered dose was exhaled as $^{14}CO_2$ or other volatiles during a sampling period of 48 hours. This demonstrated the stability of the pyridinylmethyl-^{14}C label with regard to formation of volatile products.

In male rats, maximum radioactivity concentrations (CEQ_{max} expressed as µg a.s. equiv. /g) were reached for almost all organs and tissues at one hour after administration (t_{max}). At this time, the values for liver and kidney were significantly higher than in blood, suggesting a preferred clearance from blood and distribution mainly to these organs which are responsible for metabolism (liver) and excretion (kidney). Higher levels than in blood were also detected in the myocardium (heart) and in several glands. Very low levels were found in the brain, spinal cord and in renal fat.

In female rats, maximum concentrations were also reached for almost all organs and tissues at one hour after administration (t_{max}). At this time, the values for liver and kidney were also significantly higher than in blood as found for males. The distribution in the other organs and tissues was also similar to the male animals.

After reaching the peak concentrations, a fast decline of radioactivity concentrations was observed for all organs and tissues in males and females during the entire test period. Concentrations fell for most organs and tissues below 5% of the maximum after one day and below the limit of quantification after seven days post administration. After seven days, only minor to very low concentrations of radioactivity were found in a few organs and tissues.

Basically males and female rats exhibited a very similar absorption, distribution and excretion behavior. Based on the results of this study, any accumulation or significant retention of [pyridinylmethyl-^{14}C] BYI 02960 in male and female rats can be excluded.

I. Material and Methods

A. Material

1. Test Material:

IUPAC Name:	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name:	BYI 02960
Common name:	Flupyradifurone
Empirical formula:	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass:	288.68 g/mol
Water solubility:	pH 4 = 3.2 mg/L, pH 7 = 3.2 mg/L, pH 9 = 3.0 mg/L
n-Octanol/water partition coefficient:	pH 4 = 1.2, pH 7 = 1.2, pH 9 = 1.2
Labelling:	[pyridinylmethyl-^{14}C]
Specific radioactivity of the radiolabelled batches:	4.37 MBq/mg = 2.622 x 10^5 dpm/µg = 118.08 µCi/mg = 34.09 Ci/mol
Specific radioactivity used for administration:	4.37 MBq/mg = 2.622 x 10^5 dpm/µg = 118.08 µCi/mg = 34.09 Ci/mol
Radiochemical purity:	> 99 % (certified, HPLC and TLC with radiodetection)
Dose level:	5 mg/kg body weight
Vehicle:	0.5 % aqueous tragacanth suspension
Stability of the test material:	The stability of [pyridinylmethyl-^{14}C]BYI 02960 was determined by radio-HPLC of the administration suspensions of each test immediately after dosing

2. Test Animals:

Species:	Rat (*Rattus norvegicus domesticus*)	
Strain:	Wistar Hsd/Cpb: WU	
Breeding facility:	[redacted]	
Sex and numbers involved:	Males:	8 + 1 control animal
	Females:	8 + 1 control animal
	control animals were dosed with non radiolabelled test substance	
Age:	5 weeks (male rats) and 8 weeks (female rats) at the time of delivery	
Body weight:	Males:	202 - 206 g at the time of administration
		196 - 228 g at the time of sacrifice
	Females:	187 - 210 g at the time of administration
		189 - 219 g at the time of sacrifice
Acclimatization:	Makrolon® cages on wood shavings in the test facility for about 7 days prior to the administration.	
Identification:	Cage cards on which the study number, test compound name and individual animal number were displayed. Additional labelling with water-insoluble spots on the tail.	
Housing:	After administration of the radiolabelled test item individually in Makrolon® metabolism cages under conventional hygienic conditions in air-conditioned rooms Temperature 19 - 24 °C, relative humidity 49 - 61 % 12/12 hours light / dark cycle, air change 10 - 15 times per hour	
Feed and water:	Rat/mice maintenance long life diet (no. 3883.0.15), supplied by Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland (ca. 16 g per animal and day) last feeding ca. 16 h prior to dosing next feeding ca. 6 h after dosing tap water from municipal water supply, *ad libitum*	

B. Study Design

1. Dosing

Each of the rats (8 rats per gender) orally received 5 mg/kg bw of [pyridinylmethyl-^{14}C]BYI 02960 suspended in 0.5 % aqueous tragacanth after ca. 16 h of fasting. A control animal of each gender was treated with 5 mg/kg bw non-radiolabelled BYI 02960. The dosing suspensions (0.3 mg/mL) were prepared one day before dosing and stored below 4°C.

The suspensions were administered by oral gavage using a syringe attached to an animal-feeding knob cannula. Each animal received a volume of 2 mL (10 mL/kg bw calculated for a nominal body weight of 200 g). The amounts of the administered test substance were calculated by dividing the administered radioactivity amounts by the specific radioactivity. The actual mean administered dose of [pyridinylmethyl-^{14}C]BYI 02960 was 4.89 mg/kg bw for male rats and 5.27 mg/kg bw for female rats. The stability of the test compound in the suspensions was assured by radio-HPLC analysis after administration.

2. Collection of excreta

After administration of the radiolabelled test compound, the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine, faeces, and expired air. Urine was collected separately for each animal in a cryogenic trap cooled with dry ice in intervals of 1h, 4 h, 8 h, 24 h, and every 24 h until 168 h. The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction. The radioactivity was determined by LSC.
The faeces samples were collected every 24 h separately for each animal before they were lyophilised, weighed, and homogenised. The radioactivity was determined by combustion/LSC.

3. Trapping of expired air

Carbon dioxide and other volatiles from expired air were collected from four male and four female animals for the time ranges 0 - 24 h and 24 - 48 h. The respective metabolism cages were attached to a high velocity air pump and ventilated with ca. 2 L of air per minute and cage. The air was passed through a trapping system of two gas-washing bottles each containing about 150 - 200 mL of a 1:1-mixture of ethanolamine/ethanol. At sampling, the exact volume was determined, from which an aliquot was taken for the determination of radioactivity by LSC.

4. Sacrifice and preparation of carcass for autoradiography

One animal of each gender was sacrificed using carbon dioxide at 1, 4, 8, 24, 48, 72, 120, and 168 h post administration, respectively. The control animals were sacrificed 4 h after dosing.
After sacrifice, the animals were fixed in a stretched position using a metal template and immediately frozen at ca. -70 °C in a dichloromethane/dry ice bath. After removal of the template, the animal body together with a series of blood standards (^{14}C labelled compound in bovine blood) was embedded in a slurry of carboxymethylcellulose (7 to 8 %) on the platform of the cryomicrotome (Leica CM 3600, Leica Instruments GmbH, 69226 Nussloch/ Germany).

5. Autoradiography

Sagittal sections of 50 µm thickness were cut at ca. -25 °C using a cryomicrotome, attached to adhesive tape, and lyophilised overnight in the cooling cabinet of the microtome. Four to five sections exhibiting the relevant organs and tissues were prepared from each animal. All cryosections were exposed at approx. +4 °C in a lead shielding box in order to minimize the background signal for 6 to 120 hours before being scanned with the Fuji BAS 5000™ image analyzer. The sections were stored at about -20 °C at all times except during exposure.
The sections of the control animals were exposed under identical conditions, using the longest exposure time which had been chosen for rat sections of animals treated with the radiolabelled compound. No blackening of typical animal structures could be observed after an exposure time of 120 hours for male and female animals.
The digital images of the radioluminograms allowed for the assessment of the radioactivity concentration distribution in different organs and tissues. The autoradiograms were quantitatively evaluated using the AIDA® software (Raytest, Straubenhard, Germany). Defined areas were set and integrated in each organ or tissue or substructure thereof. After background subtraction, a value of the photostimulated luminescence (PSL) per mm^2 was obtained, which is proportional to the equivalent concentration of the radioactivity in that particular tissue.

Two series of 8 calibration standards were prepared by spiking bovine control blood with different concentrations of a ^{14}C-radiolabelled reference compound. The concentrations covered a range from approx. 400 to 2,000,000 dpm/g. The radioactivity of each blood calibration standard was determined by combustion/LSC and the mean values of each standard were used to establish a calibration graph for the correlation of (PSL - Bkg)/mm^2 to the radioactivity in dpm/g tissue by linear regression analysis. The obtained regression factors were used to calculate the concentration of the radioactivity in dpm/g in the lyophilised rat sections. These values were corrected for the self-absorption of the various types of tissues using self absorption factors published in the literature. To express the values as equivalent concentrations, the radioactivity concentrations given in dpm/g were divided by the specific radioactivity of the test substance in dpm/µg.

6. Radioactivity measurement

The measurement of radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). All solid samples and blood standards were weighed and combusted in an oxygen atmosphere with an Oxidizer 307/387 (Packard Instruments).
For all samples, the limit of detection (LOD) was established at ca. 20 dpm measured per aliquot after correction for the background radioactivity. The limit of quantification (LOQ) for each individual measurement was established as 2 times of the background radioactivity (dpm) of each instrument/method. This background counting rate was in a range between 12 - 30 cpm (approximately 12 - 30 dpm), it was automatically subtracted from the measured results. A quench and counting efficiency correction for transformation of gross counts (cpm) into net counts (dpm) was automatically performed by the instruments.

II. Results and Discussion

A. Distribution

The distribution of BYI 02960 in male and female rats was investigated by quantitative whole body autoradiography (QWBA) using the radioluminography (RLG) technique. The data were obtained over a test period of 7 days in male and female rats following a single oral administration of radiolabelled [pyridinylmethyl-^{14}C]BYI 02960 at a dose level of 5 mg/kg body weight. Eight male and female rats each were treated and sacrificed at 1, 4, 8, 24, 48, 72, 120 and 168 hours after dosing.
For almost all organs and tissues, maximum concentrations were reached at one hour after administration. At t_{max}, the organ/blood concentration ratios for many organs and tissues were in a range of approx. 0.8 - 1.2, *i.e.* the radioactivity concentration in these organs and tissues was in the range of ± 20% the concentration in blood.
The radioactivity administered with [pyridinylmethyl-^{14}C]BYI 02960 was rapidly cleared from the blood and distributed to the entire body, preferably to those organs or tissues responsible for metabolism (liver), excretion (kidney) and secretion (*e.g.* adrenal, thyroid, Harderian and salivary glands). The lowest values were measured for perirenal fat, the spinal cord, and brain.
The tissue/blood-concentration ratios were very similar in both sexes and highest for the liver (factor 1.78) followed by adrenal gland (1.76), and kidney (1.68). Ratios for myocardium, thyroid, Harderian gland, salivary gland, and pancreas were in the range of approx. 1.2 - 1.4. The lowest values were calculated for the spinal cord (0.37), brain (0.35), and perirenal fat (0.15).

The equivalent concentrations in blood, organs and tissues declined rapidly following approximately first order kinetics in the time range between 1 and 48 hours. At 24 hours after dosage, the concentrations in blood and also in most other tissues were below 5% of the maximum concentration, except nasal mucosa and vitreal body with approx. 7 - 11% of CEQ_{max}. At 48 hours after administration the concentrations had declined to below 1% of the maximum concentration, except vitreal body and nasal mucosa which exhibited approx. 3 - 8% of $CEQ_{max.}$ All factors are average values of male and female rats, respectively.
After seven days, the equivalent concentrations in almost all organs and tissues had fallen below the limit of quantification. Very low concentrations of radioactivity were still detectable in blood, renal medulla, liver, and lung. For all these organs, the concentration was lower than in the blood. The results are summarized in Table 5.1.1-08 (male rats) and Table 5.1.1-09 (female rats) below.

B. Excretion

BYI 02960 was excreted rapidly and completely within ca. 48 hours. The major part of the radioactivity (up to 85% in male and up to 93% in female rats) was excreted with urine and the minor part (minimum ca. 20% for male and ca. 8% for female rats) with faeces. One day after dosing >90% were excreted and the excretion was almost complete after two days.
The expiration of ^{14}C-carbon dioxide and other ^{14}C-labelled volatiles was tested with male and female animals for a test period of 48 hours. Less than 0.1 % of the administered dose was expired during the sampling period. This demonstrates the stability of the pyridinylmethyl-^{14}C label with regard to possible formation of volatile products. The excretion behavior is summarized in Table 5.1.1-10 (male rats) and Table 5.1.1-11 (female rats).

III. Conclusions

The new insecticide BYI 02960, labelled with ^{14}C in the pyridinylmethyl-position, was readily absorbed from the gastrointestinal tract of male and female rats after single oral administration. The radioactivity was distributed throughout the body immediately after administration but with a clear preference to liver and kidney.
Peak concentrations of radioactivity for almost all organs and tissues were reached already one hour after dosing. From then onwards, the concentrations declined rapidly and fell for most organs and tissues below 5% of the maximum after one day and below the limit of quantification after seven days post administration.
The radioactivity was quickly excreted, mainly with the urine. More than 90% of the administered dose was excreted within 24 hours, and 95 to 100% after 48 hours.
Female rats showed a slightly higher proportion of renal excretion (89 - 93% of administered dose) as compared to male animals (72 - 85%). In general, males and females had a very similar absorption, distribution and excretion behavior.
Based on the results of this study, any accumulation or significant retention of [pyridinylmethyl-^{14}C] BYI 02960 in male and female rats can be excluded.

Table 5.1.1-08: Distribution of radioactivity in organs and tissues of male rats after a single oral dose of 5 mg/kg [pyridinylmethyl-^{14}C]BYI 02960

Organ or tissue	Equivalent concentration CEQ [µg a.s. equiv./g]							
	Time of sacrifice [hours after administration]							
	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h
Blood	3.171	2.376	1.692	0.122	0.026	0.020	0.013	0.009
Liver	5.645	4.326	3.332	0.246	0.027	0.014	0.008	0.005
Renal cortex	4.441	3.267	2.565	0.146	0.016	0.007	0.005	<LOQ
Renal medulla	6.398	4.538	3.680	0.248	0.021	0.013	0.008	0.006
Kidney total	5.420	3.903	3.122	0.197	0.019	0.010	0.006	0.005
Brown fat	3.288	2.479	2.023	0.098	0.007	---	---	---
Perirenal fat	0.446	0.351	0.219	0.013	<LOQ	<LOQ	<LOQ	<LOQ
Skeleton muscle	3.246	2.283	1.608	0.096	0.005	<LOQ	<LOQ	<LOQ
Myocardium	4.426	3.155	2.250	0.142	0.007	<LOQ	<LOQ	<LOQ
Lung	2.450	2.168	1.373	0.082	0.015	0.013	0.005	0.005
Spleen	2.873	2.093	1.557	0.096	0.010	<LOQ	<LOQ	<LOQ
Pancreas	4.089	2.882	2.170	0.129	0.005	<LOQ	<LOQ	<LOQ
Bone marrow	2.770	1.825	1.391	0.083	---	---	---	---
Testis	2.251	2.198	1.466	0.092	<LOQ	<LOQ	<LOQ	<LOQ
Brain	1.214	0.852	0.619	0.036	<LOQ	<LOQ	<LOQ	<LOQ
Spinal cord	1.334	1.004	0.771	0.042	<LOQ	---	---	---
Pituitary gland	3.539	2.435	1.889	0.105	---	---	---	---
Pineal body	3.033	1.488	1.680	0.089	0.005	<LOQ	---	---
Adrenal gland	5.626	4.429	3.338	0.213	0.017	0.007	<LOQ	<LOQ
Thymus	3.173	2.328	1.646	0.096	<LOQ	<LOQ	<LOQ	<LOQ
Thyroid gland	4.252	3.284	2.219	0.143	0.012	<LOQ	---	---
Salivary gland	4.123	3.097	2.195	0.131	0.008	<LOQ	<LOQ	<LOQ
Nasal mucosa	1.409	1.477	1.210	0.250	0.120	0.077	0.043	0.037
Vitreal body	1.575	1.370	1.459	0.273	0.073	0.036	0.011	<LOQ
Harderian gland	4.469	3.083	2.415	0.151	0.014	---	---	---

--- : Organ or tissue was visible in the rat sections but not discernible in the radioluminograms
< LOQ : below limit of quantification

Table 5.1.1-09: Distribution of radioactivity in organs and tissues of female rats after a single oral dose of 5 mg/kg [pyridinylmethyl-^{14}C]BYI 02960

Organ or tissue	Equivalent concentration CEQ [µg a.s. equiv. /g]							
	Time of sacrifice [hours after administration]							
	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h
Blood	4.155	3.211	2.005	0.090	0.016	0.012	0.010	0.007
Liver	7.451	5.878	3.778	0.171	0.016	0.008	0.006	<LOQ
Renal cortex	5.460	4.414	2.834	0.124	0.009	0.005	0.005	<LOQ
Renal medulla	8.212	7.083	4.877	0.222	0.013	0.009	0.007	0.008
Kidney total	6.836	5.748	3.856	0.173	0.011	0.007	0.006	0.006
Brown fat	3.229	3.174	1.811	0.095	<LOQ	---	---	---
Perirenal fat	0.413	0.502	0.315	0.019	<LOQ	<LOQ	<LOQ	<LOQ
Skeleton muscle	3.746	3.140	1.926	0.075	<LOQ	<LOQ	<LOQ	<LOQ
Myocardium	5.198	4.474	2.701	0.105	0.006	<LOQ	<LOQ	<LOQ
Lung	3.230	1.142	1.311	0.054	0.008	0.007	0.005	0.005
Spleen	3.742	2.975	1.952	0.077	<LOQ	<LOQ	<LOQ	<LOQ
Pancreas	5.245	3.961	2.626	0.100	<LOQ	---	<LOQ	<LOQ
Bone marrow	2.843	2.344	1.476	0.062	---	---	---	---
Ovary	3.688	3.101	1.915	0.077	0.006	<LOQ	<LOQ	---
Uterus	3.950	3.262	2.052	0.081	<LOQ	<LOQ	<LOQ	<LOQ
Brain	1.279	1.052	0.626	0.026	<LOQ	<LOQ	<LOQ	<LOQ
Spinal cord	1.348	1.074	0.692	0.029	<LOQ	---	---	---
Pituitary gland	4.306	3.793	2.293	0.082	---	---	---	---
Pineal body	3.427	2.797	1.914	0.075	---	---	---	---
Adrenal gland	7.289	5.669	4.029	0.201	0.013	0.008	<LOQ	<LOQ
Thymus	4.090	3.180	1.971	0.075	<LOQ	<LOQ	<LOQ	<LOQ
Thyroid gland	5.548	4.284	2.809	0.102	0.006	---	---	---
Salivary gland	5.411	4.177	2.642	0.102	0.006	<LOQ	<LOQ	<LOQ
Nasal mucosa	1.585	2.381	1.720	0.164	0.102	0.068	0.047	0.037
Vitreal body	2.303	2.062	1.276	0.248	0.062	0.024	0.009	0.008
Harderian gland	5.006	4.048	2.483	0.107	0.019	<LOQ	---	---

--- : Organ or tissue was visible in the rat sections but not discernible in the radioluminograms
< LOQ : below limit of quantification

Table 5.1.1-10: Excretion of radioactivity in urine, faeces and expired air of male rats after a single oral administration of 5 mg/kg [pyridinylmethyl-^{14}C]BYI 02960

	Percent of radioactive dose administered							
	Time of sacrifice [h after administration]							
	1	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Exhaled air								
24 h					0.06	0.06	0.07	0.06
48 h					0.07	0.08	0.09	0.09
Urine								
1 h	2.38							
4 h		22.39	12.55	14.62	2.71	2.16	2.03	11.86
8 h			42.07	45.19	23.14	30.59	44.03	29.61
24 h				78.07	68.52	79.11	76.13	75.12
48 h					72.34	84.54	79.85	79.67
72 h						85.12	80.35	80.05
96 h							80.53	80.19
120 h							80.70	80.29
144 h								80.38
168 h								80.50
Faeces								
24 h	*	*		14.93	23.63	17.37	20.26	17.49
48 h					26.75	20.34	22.49	21.59
72 h						20.54	22.74	21.92
96 h							22.82	21.98
120 h							22.85	22.04
144 h								22.05
168 h								22.12
Sum total	**2.38**	**22.39**	**42.07**	**92.99**	**99.17**	**105.74**	**103.64**	**102.71**

* : faeces not collected

Table 5.1.1-11: Excretion of radioactivity in urine, faeces and expired air of female rats after a single oral administration of 5 mg/kg [pyridinylmethyl-^{14}C]BYI 02960

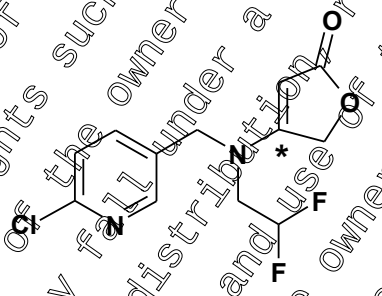
	Percent of radioactive dose administered							
	Time of sacrifice [h after administration]							
	1	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Exhaled air								
24 h					0.02	0.02	0.02	0.02
48 h					0.03	0.03	0.02	0.03
Urine								
1 h	7.19							
4 h		9.79	9.69	12.35	11.53	3.55	25.51	4.35
8 h			32.74	33.40	27.68	49.84	26.53	4.41
24 h				86.47	84.60	86.82	90.42	78.34
48 h					89.90	88.76	92.95	88.13
72 h						88.94	93.16	89.93
96 h							93.23	91.15
120 h							93.28	91.61
144 h								91.95
168 h								92.46
Faeces								
24 h	*	*		10.62	8.61	8.75	6.70	4.54
48 h					10.58	9.75	7.82	6.98
72 h						9.78	7.89	7.36
96 h							7.92	7.47
120 h							7.93	7.49
144 h								7.52
168 h								7.53
Sum total	**7.19**	**9.79**	**32.74**	**97.09**	**100.50**	**98.76**	**101.23**	**100.03**

* : faeces not collected

KIIA 5.1.2 - Toxicokintic studies - Second single dose, oral route, in rats

Three studies are summarised in this chapter. The first study report (No. MEF-11/556) describes the absorption, distribution, metabolism, and excretion of the furanone-4-^{14}C labelled test compound in male and female rats which were dosed with a single low dose. The excretion of radioactivity was determined in urine and faeces, the radioactivity concentration was determined in the organs and tissues at sacrifice and the metabolites were identified in the excreta. The toxicokinetic behaviour of the total radioactivity was investigated by plasma curve analysis.
The second study (Report No. MEF-11/275) describes the distribution and excretion of the furanone-4-^{14}C labelled test compound in male and female rats after a single oral low dose. In this study, the distribution at different time points was measured by quantitative whole body autoradiography. Excretion was investigated via urine, faeces and air expired.
The third study (Report No. MEF-11/271) describes the distribution and excretion of the furanone-4-^{14}C labelled test compound in male and female rats after a single oral low dose. The distribution of the total radioactivity was determined in urine as well as in plasma, liver, kidney, muscle and fat at sacrifice. The metabolism was investigated in urine, plasma, and in extracts of liver, kidney, muscle, and fat.

The position of the radiolabel is shown in this figure:

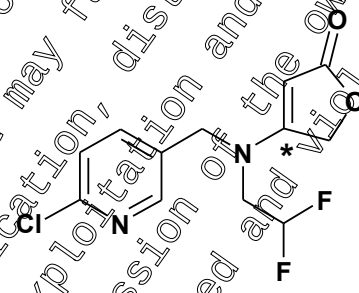


[furanone-4-^{14}C]BYI 02960

Report:	**KIIA 5.1.2/01, [redacted]; 2011**
	[Furanone-4-^{14}C]BYI 02960 - Absorption, Distribution, Excretion, and Metabolism in the Rat
Report No & Document No	MEF 11/556 M-421499-01-1
Guidelines:	OECD Guideline for Testing Chemicals, 417: Toxicokinetics US EPA Health Effects Test Guidelines OPPTS 870.7485, Metabolism and Pharmacokinetics PMRA Regulatory Directive Dir2005-01: Section 11: Toxicokinetic studies Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	Yes, fully compliant US EPA – FIFRA Good Laboratory Practice (40 CFR Part 160) Principles of Good Laboratory Practice - German Chemical Law (Chemikaliengesetz), dated 2002-06-20, current version of Annex 1 JAPAN MAFF - Notification on the Good Laboratory Practice Standards for Agricultural Chemicals (11 Nousan 6283) notified 1999-10-01
Testing Facility and Dates	[redacted] Experimental work: 2009-05-1 - 2011-06-20

Executive Summary

The absorption, distribution, excretion, and metabolism of the new insecticide BYI 02960 (common name: flupyradifurone) were investigated in male and female Wistar rats. Groups of four rats were administered orally by gavage with a single dose of BYI 02960 in 0.5% aqueous Tragacanth® at a dose level of 2 mg/kg bw. The test compound was radiolabelled with ^{14}C in the 4-position of the furanone ring of the molecule as shown below:



• **denotes the position of the ^{14}C-label**

The animals were sacrificed 168 h after dosing. Urine and faeces were collected in intervals during the test period. The radioactivity level in plasma was followed by collection of micro samples from each animal for the whole test period (23 time points). The total radioactivity was determined in excreta and in organs and tissues collected at sacrifice. Metabolism was investigated in urine and faeces.
The total recovery was almost quantitative since approx. 100% of the administered dose was found in the excreta and in the body at sacrifice. [Furanone-4-^{14}C]BYI 02960 was almost completely absorbed since >79% of the dose administered was detected in the urine and the body without GIT at sacrifice of male and female rats. The absorption commenced immediately after dosing as can be seen from the fast increase of radioactivity in the plasma.

The distribution of the radioactivity within the body was fast and the maximum plasma level (C_{max}) was reached within 1.5 hours after administration. Then, the radioactivity level declined slowly to ca. 50% of C_{max} after 8 hours and to <1% of C_{max} 72h after dosing.
Excretion was fast and mainly renal. Approx. 79% of the dose was found the urine of male rats and approx. 91% in the urine of female animals. The major part of the dose was excreted within 24 hours after treatment by male and female rats. At the time of sacrifice a small proportion of ca. 0.5% of the dose for males and ca. 0.2% for females was still detected in the body without GIT.
Metabolic profiles in urine and extracts of faeces were determined by reversed phase HPLC with radiodetection using a neutral water/acetonitrile gradient.
Parent compound, one major and six minor metabolites were identified by HPLC comparison and LC-MS/MS of an isolated and purified compound. Identification rates were >84% of the total dose in male rats and >95% in females. Another ca. 8% of the dose in males and 4% in females was characterized by their chromatographic behavior. All metabolites accounting for >1% of the dose administered were identified except a polar metabolite fraction accounting for 5.30% in males and 2.45% in females. This polar fraction most probably consisted of labelled biomolecules. The identified metabolites in the excreta, expressed as % of the total dose, are summarized in the Table 5.1.2-01:

Table 5.1.2-01: Amounts of identified metabolites expressed as % of the total dose in the excreta of rats after oral administration of 2 mg/kg [furanone-4-^{14}C]BYI 02960

	Male 2 mg/kg	Female 2 mg/kg
Parent compound	54.68	75.96
difluoroethyl-amino-furanone	3.49	0.96
BYI 02960-OH-gluA (isomer 1)	1.13	0.49
BYI 02960-OH-gluA (isomer 3)	2.17	1.01
BYI 02960-des-difluoroethyl	2.12	3.35
BYI 02960-OH-SA	0.24	0.34
BYI 02960-OH	20.60	13.25
BYI 02960-iso-OH	0.26	0.07
Total identified	**84.70**	**95.42**
Total characterized	**8.00**	**4.08**
Total	**95.55**	**101.75**

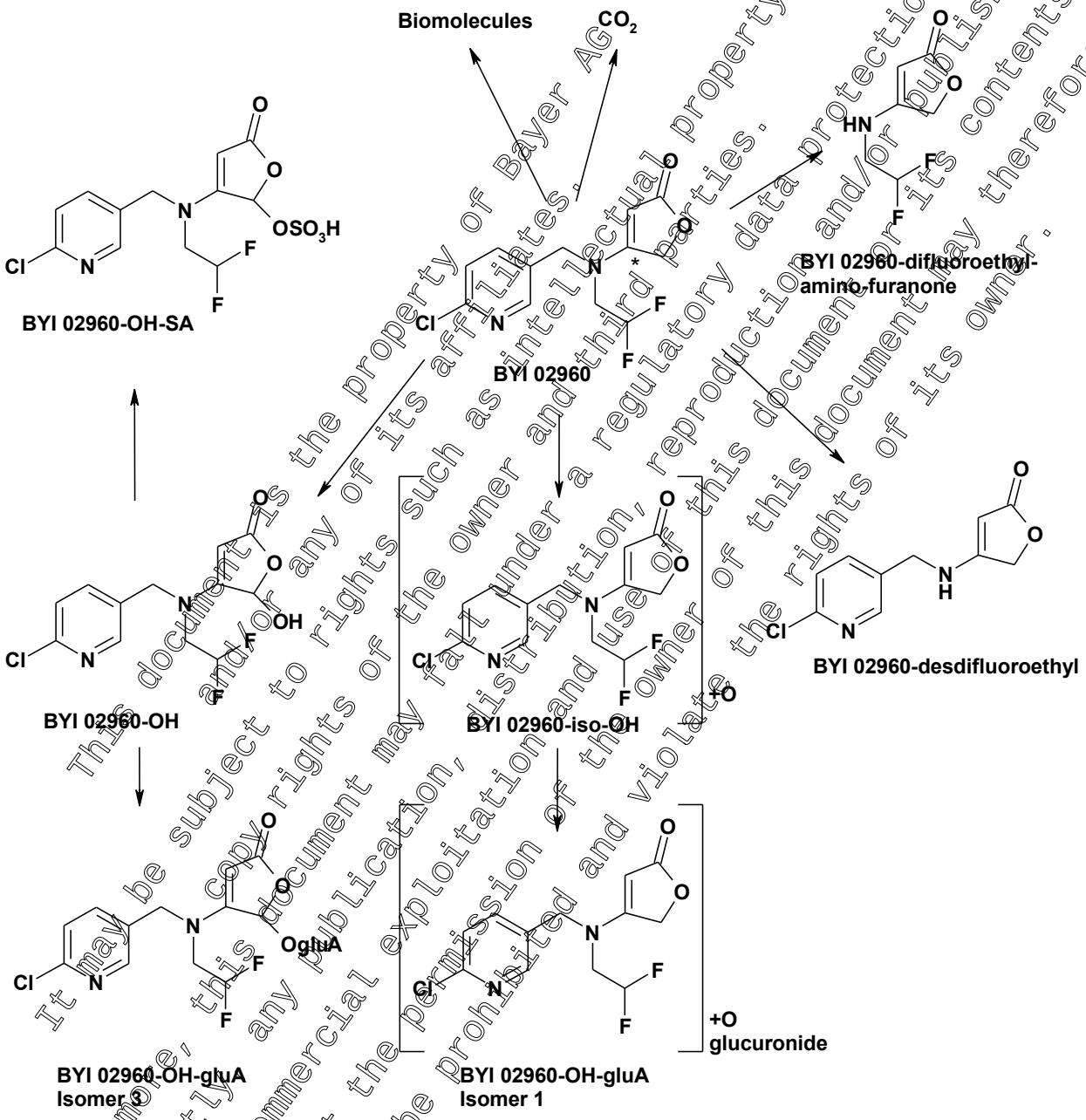
Basically, male rats showed a higher rate of metabolism with only ca. 55% of unchanged parent compound found in excreta whereas 74% of unchanged BYI 02960 was found in the excreta of female rats. Nevertheless, the metabolic patterns were very similar in males and females.
The principal metabolic reactions of [furanone-4-^{14}C]BYI 02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid or sulfate,
- cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl, and
- cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

Male rats exhibited a higher proportion of the polar metabolite fraction and higher residues in the body at the time of sacrifice as well as a higher proportion of radioactivity in exhaled air. All these observations suggest that the furanone-4-^{14}C radiolabel is not completely stable and that a small part of

the dose obviously underwent extensive biotransformation to C1- and C2-fragments which were incorporated into biomolecules, particularly in male rats.
The results of these metabolism investigations are in very good accordance with those obtained in the corresponding organ metabolism rat study (KIIA 5.1.2/03) and the ADME study using the pyridinylmethyl-label (KIIA 5.1.1/01).

The proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in rats is shown below:



*** position of ^{14}C label**

I. Material and Methods

A. Material

1. Test Material:

IUPAC Name:	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name:	BYI 02960
Common name:	Flupyradifurone
Empirical formula:	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass:	288.68 g/mol
Water solubility:	pH 4 = 3.2 mg/L, pH 7 = 3.2 mg/L pH 9 = 3.0 mg/L
n-Octanol/water partition coefficient:	pH 4 = 1.2, pH 7 = 1.2, pH 9 = 1.2
Labelling:	[furanone-4-^{14}C]
Specific radioactivity of the radiolabelled batches:	3.94 MBq/mg = 2.36 x 10^5 dpm/µg = 106.46 µCi/mg = 30.73 Ci/mol
Specific radioactivity used for administration:	3.94 MBq/mg = 2.36 x 10^5 dpm/µg = 106.46 µCi/mg = 30.738 Ci/mol
Radiochemical purity:	> 99 % (certified, HPLC and TLC with radiodetection)
Dose level:	2 mg/kg body weight
Vehicle:	0.5 % aqueous tragacanth suspension
Stability of the test material:	The stability of [ethyl-1-^{14}C]BYI 02960 was determined by radio-HPLC of the administration suspensions immediately after dosing

2. Test Animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	Wistar Hsd/Cpb: WU
Breeding facility:	[redacted]
Sex and numbers involved:	4 male animals
Age:	Males: ca. 6 weeks at the time of delivery Females: ca. 8 - 9 weeks at the time of delivery
Body weight:	202 - 212 g at the time of administration
Acclimatization:	Makrolon® cages on wood shavings in the test facility for 7 days prior to the administration.
Identification:	Cage cards on which the study number, test compound name and individual animal number were displayed. Additional labelling with water-insoluble spots on the tail
Housing:	After administration of the radiolabelled test compound individually in Makrolon® metabolism cages under conventional hygienic conditions in air-conditioned rooms Temperature 22 - 24 °C, relative humidity 45 - 67 %. 12/12 hours light / dark cycle, air change 10 - 15 times per hour

Feed and water:	Rat/mice maintenance long life diet (no. 3883.0.15), supplied by Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland (ca. 16 g per animal and day) Last feeding ca. 16 h prior to dosing Next feeding ca. 6 h after dosing tap water from municipal water supply, *ad libitum*

B. Study Design

1. Dosing

Each rat orally received 2 mg/kg of [furanone-4-^{14}C]BYI 02960 suspended in 0.5 % aqueous tragacanth after ca. 16 h of fasting. The dosing suspension (0.21 mg/mL) was prepared in a cold room at 5°C. The suspension was administered by oral gavage using a syringe attached to an animal-feeding knob cannula. Each animal received a volume of 2 mL (10 mL/kg bw calculated for a nominal body weight of 200 g). The individual amounts of the administered test substance were calculated by dividing the amount of the administered radioactivity by the specific radioactivity. The actual mean administered dose of [furanone-4-^{14}C]BYI 02960 was 2.0 mg/kg. The stability of the test compound in the suspension was assured by radio-HPLC analysis after administration.

2. Collection of excreta

After administration the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine, faeces, and expired air. Urine was collected separately for each animal in a cryogenic trap cooled with dry ice in intervals of 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 96h, 120 h, 144 h and 168 h. The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction. The radioactivity was determined by LSC.
The faeces samples were collected every 24 h separately for each animal before they were lyophilised, weighed, and homogenised. The radioactivity was determined by combustion/LSC.

3. Plasma micro-samples

Blood samples were collected separately for each animal by pressing a capillary coated with heparin in a small cut in the tail vein. The wound was sealed with adhesive tape. The capillaries were centrifuged using a hematocrit centrifuge to separate plasma from the formed blood constituents. After centrifugation, the capillary was broken between plasma and formed constituents and the plasma (approx. 10 - 100 mg) squeezed out onto a small metal dish for weighing. After weighing, this dish was placed into a scintillation vial for radioactivity measurement. Blood samples were collected at 10 min, 20min, 40 min, 1h, 1.5h, 2h, 3h, 4h, 6h, 8h, 24h, 28h, 32h, 48h, 52h, 56h, 72h, 96h, 120h, 144h, 152h and 168h after dosing from the same animals and plasma curves could thus be generated for single animals avoiding inter-animal variations. For pharmacokinetic calculations, the average plasma value of the four rats was used.

4. Sacrifice

The rats were sacrificed in Pentobarbital-Na anaesthesia (Narcoren®, supplied by [redacted], Germany) by transection of the cervical vessels and exsanguinated.

5. Plasma, tissues and organs at sacrifice

At sacrifice, blood was collected in heparinised test tubes and separated into plasma and blood cells by centrifugation. After weighing, aliquots were taken for determination of radioactivity by LSC. Organs and tissues were weighed immediately after dissection and again after lyophilisation. Finally they were homogenized prior to taking aliquots for the determination of radioactivity by combustion/LSC. For small organs and tissues such as adrenal glands, thyroid, renal fat, only the wet weight was determined before they were solubilised using BTS 450 (Beckman Tissue Solubiliser) and radioassayed by LSC. The radioactivity remaining in the GIT, skin, and residual carcass was determined by combustion/LSC in order to establish the radioactivity balance. The gastro-intestinal tract and an aliquot of depilated skin were lyophilised. After weighing and homogenization, aliquots were taken for determination of radioactivity by combustion/LSC.

6. Measurement of radioactivity

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). All solid samples were combusted in an oxygen atmosphere using an oxidizer. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

7. Toxicokinetic analysis

In this study the software TOPFIT (ver. 2.0) was used to calculate the toxicokinetic parameters by plasma concentration-time curve analysis for the mean equivalent concentration values. A standard 2-compartment disposition model was applied for curve fitting. Compartment models attempt to mathematically describe the processes of absorption, entry into the systemic circulation, distribution to organs or tissues where metabolism can occur, and subsequent excretion.

8. Analytical methods

Samples were analyzed by radiochromatographic (HPLC, TLC) and spectroscopic (LC-MS, ^{1}H-NMR) methods.

9. High Performance Liquid Chromatography (HPLC)

HPLC-radioactivity analysis was performed on an Agilent 1100 system with radiometric- and UV-detection. The separation was carried out on a reversed phase column using a neutral water / acetonitrile gradient. Detailed information of the HPLC-methods and instruments set-up can be found in the report. All solvents were of HPLC-quality.

The chromatograms were recorded and quantitatively evaluated using the software package GINA® (raytest, Benzstraße 4, D-75334 Straubenhardt, Germany). The ^{14}C-trace of a chromatogram was divided into regions of interest (ROI's), according to the separated radioactive peaks. Additionally, background regions (BKG's) were defined and used for background correction. The background corrected area counts from all regions of interest were used for calculation of the quantitative distribution of the individual components in the sample. A quantifiable radioactive peak was regarded relevant if resulting in a signal approx. 2.5 times above the background noise.

For co-chromatography in HPLC, the sample was mixed with the reference compound before injection. The detection was achieved either by UV-absorption of the non-radiolabelled or by ^{14}C-detection of the radiolabelled reference compound. Chromatographic matching with the non-radiolabelled reference compound was assessed by comparison of the UV-trace and the associated ^{14}C-trace. Chromatographic matching with the radiolabelled reference compound was assessed by comparison of the ^{14}C-chromatogram of the mixture with the ^{14}C-chromatogram of the sample without the reference compound.

10. Thin Layer Chromatography (TLC)

For detection and characterization of compounds in the polar metabolite fraction (peak 1 in HPLC profile) thin-layer chromatography (silica 60 F_{254}, normal phase) was used. The samples were spotted on the TLC-plates using a Linomat IV - instrument (Camag, Berlin, Germany). The plates were developed using either acetonitrile / water / formic acid (70 / 25 / 5; v/v/v) or acetonitrile / water / ammonia solution (25%) (60 / 35 / 5; v/v/v) as a solvent system.

The TLC-bands or spots were visualized under UV-light (254 nm). The radioactive zones were detected .using a Fujibas® 2000 bio imaging system (Fuji, Japan & raytest, Straubenhard, Germany). Fujibas® imaging plates were exposed to the TLC-plates. The imaging data were identified with Basreader software (version 2.13e, raytest, Straubenhard, Germany. Evaluation and visualization of recorded data was performed with AIDA software (raytest, Straubenhard, Germany). The detection limit (LOD) was 5 - 10 dpm/zone for ^{14}C after an exposure of at least 14 hours.

For co-chromatography in TLC, a solution of the reference compound was applied to the plate as a 1 to 1.5 cm-wide band. The sample solution was also applied as a 1 to 1.5 cm-wide band, part of which (ca. 5 to 8 mm) overlapped with the reference compound band. After development, chromatographic matching with the reference compound was assessed by analysis of the individual radioluminogram.

11. Mass spectroscopy

Electro-spray ionization MS spectra (ESI) were obtained either with a TSQ 7000 instrument or with a LTQ Orbitrap XL mass spectrometer (Thermo or Finnigan, respectively, San Jose, CA, U.S.A.). The HPLC-instrument used for chromatography was an Agilent HP1100 (Agilent, Waldbronn, Germany). The eluate of the HPLC-column was split between the UV-detector followed by a radioactivity detector (Ramona Star, Raytest, Straubenhardt, Germany) and the MS spectrometer.

12. Preparation of samples, extraction and analysis

Aliquots of the urines from all four male animals were combined to a pool sample for each collection period in the time range 0 to 48 hours. These samples were used for metabolic profiling. A further pool sample of the same time range was also prepared from aliquots of the respective singe samples and used for fractionation. Urine samples of female rats for the time range 0 - 48 hours were combined and used for metabolic profiling. The amount of radioactivity applied for analysis was calculated from the in-life data of the respective samples. Aliquots of the urine samples were taken without further sample processing for metabolic profiling by HPLC. The faeces samples of the first sampling interval (0-24 h) from all four animals of a test group were combined. The level of radioactivity in the pool sample was calculated from the in-life date of the respective samples. These pool samples were extracted with acetonitrile/water mixtures. The extracts containing the major part of the radioactive residue were combined, further purified by SPE on a C18 cartridge and concentrated for subsequent HPLC analysis. The radioactivity in the post extraction solids was determined by LSC following combustion analysis.

13. Identification / Characterization and Quantification of Residues

The identification of metabolites was mainly achieved by comparison of the chromatographic profile of the urine samples with results of the organ metabolism study with [furanone-4-^{14}C]BYI 02960 (KIIA 5.1.2/03). The minor metabolites or trace metabolites BYI 02960-iso-OH and BYI 02960-OH-SA were identified by HPLC comparison with urine samples or faeces extracts from the ADME study with [pyridinylmethyl-^{14}C]BYI 02960 (KIIA 5.1.1/01). The metabolite BYI 02960-difluoroethyl-amino-furanone was isolated from urine by repeated HPLC fractionation and purification prior to high resolution LC/MS analysis. Additional characterization of the polar peak 1 (unknown 1) was performed by TLC analysis after isolation from urine. Based on the identification, all chromatograms were integrated and corresponding peaks in all samples were assigned the same peak number.

II. Results and Discussion

A. Recovery

The total recovery for both tests was almost quantitative since between approx. 96 and 102% of the administered dose was found in the excreta and the body of male and females rats at sacrifice. The somewhat lower recovery for male rats is likely due to a higher exhalation of radioactivity ($^{14}CO_2$). In the autoradiography study with [furanone-4-^{14}C]BYI 02960 (KIIA 5.1.2/02) approx. 3% of the dose was detected in expired air of male rats and approx. 1% was found for female rats. Since the amount of radioactivity in expired air was minor in the autoradiography study, a collection of air was not considered necessary in this study. The results in percent of the given dose in expired air, urine, faeces, organs and tissues at sacrifice are shown in Table 5.1.2-02 (below).

Table 5.1.2-02: Recovery of radioactivity in urine, faeces, gastrointestinal tract and the body following a single oral dose of 2 mg/kg [furanone-4-^{14}C]BYI 02960

	% of administered dose	
	Male	**Female**
Feces	16.59	10.38
Urine	78.96	91.37
Sum of excreta	**95.56**	**101.0**
Body excluding GIT	0.48	0.17
GIT	0.02	0.01
Total Body	0.49	0.18
Balance	**96.05**	**101.90**

B. Absorption

[Furanone-4-^{14}C]BYI 02960 was almost completely absorbed in male rats. The absorption rate was at least 79% in males and 91% in females because >79% or >91% of the dose was detected in urine and in the body without GIT. The absorption commenced immediately after dosing as can be seen from the quick increase of radioactivity in plasma micro samples (see Table 5.1.2-03).

Table 5.1.2-03: Timecourse of radioactivity in the plasma following oral dosing of [furanone-4-^{14}C] BYI 02960

Time [h]	Concentration [mg a.s. equiv./kg]	
	Male, 2mg/kg	Female, 2mg/kg
0.17	0.2883	0.4562
0.33	0.7373	1.1720
0.67	1.2720	1.7870
1	1.4180	1.8570
1.50	1.4570	1.9120
2	1.4460	1.8610
3	1.3570	1.7410
4	1.2550	1.5880
6	1.0220	1.2990
8	0.8145	1.0140
24	0.0621	0.0515
28	0.0461	0.0326
32	0.0375	0.0264
48	0.0168	0.0091
52	0.0162	0.0079
56	0.0149	0.0076
72	0.0113	0.0062
96	0.0087	n.d.
120	0.0066	n.d.
144	0.0048	n.d.
152	0.0048	n.d.
168	n.d.	n.d.

C. Distribution and plasma kinetics

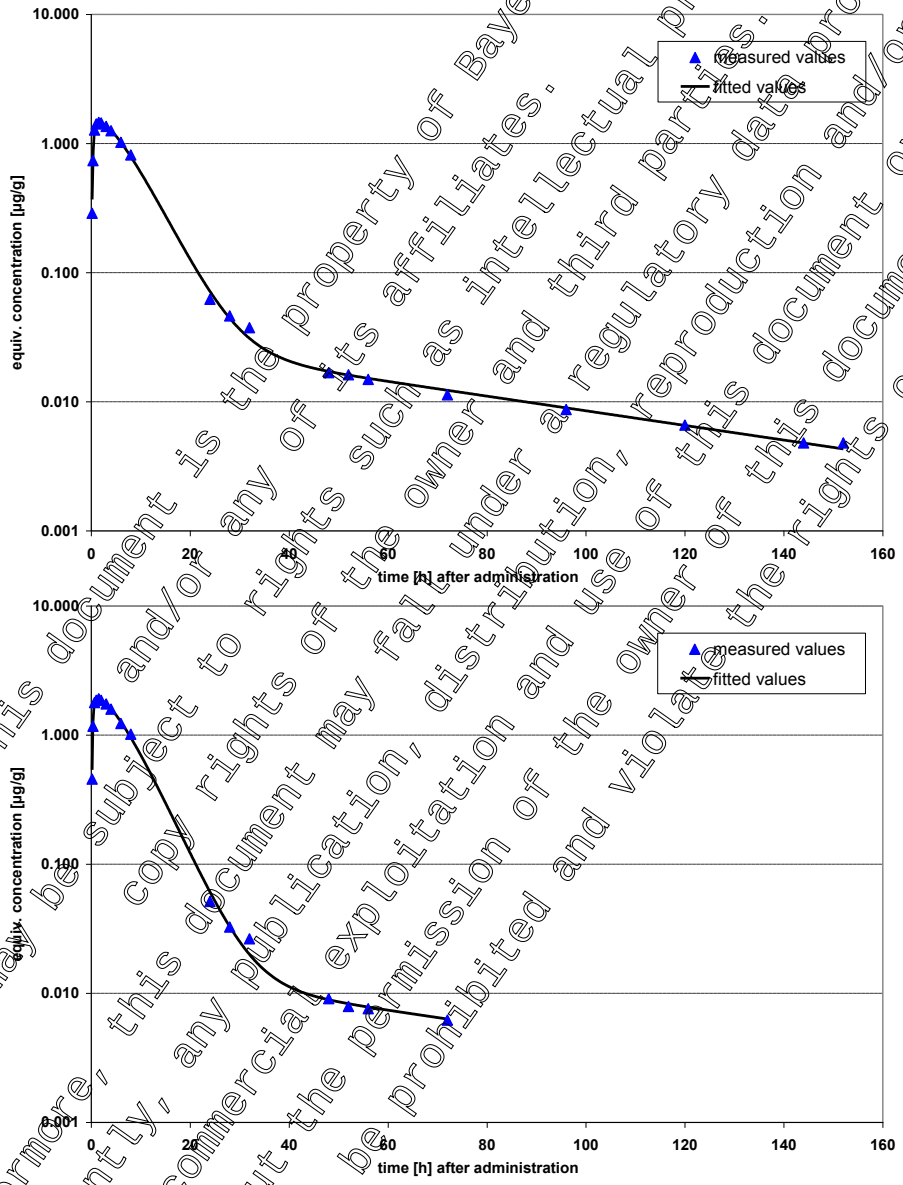
The test compound was quickly distributed as can be seen from the plasma analysis at different time points. The maximum concentration of radioactivity (C_{max}) was already reached 1.5 hours after administration. At this time, the radioactivity level in plasma corresponded approximately to the equidistribution concentration for female rats and to ca. 75% of the equidistribution concentration for male rats (see Table 5.1.2-03).

The plasma concentration then declined to approx. 50% of C_{max} within 8 hours and to approx. 2-3% of the maximum within 24 hours for both, male and female rats. Male rats exhibited about twofold higher concentrations than females in the time range ≥48 hours. This behavior is probably caused by higher amounts of [furanone-4-^{14}C]BYI 02960 metabolized to C1 and C2 fragments being incorporated into biomolecules or exhaled as $^{14}CO_2$ in males. At the time of sacrifice, 168 hours after administration, the plasma concentration was below LOQ for both, male and female animals. (see Table 5.1.2-03).

The mean values of total radioactivity were used for a toxicokinetic modelling using the TOPFIT software. A good fit could only be achieved with a three compartment model due to the obvious biphasic nature of the plasma curves. The modelling was performed for the time range between 0 and 152 hours for males and 0 to 72 hours for females. The biokinetic parameters were very similar for both

sexes but females exhibited a somewhat shorter mean residence time than males. The area under curve (AUC) was 16.0 mg/kg*h for male and 18.2 mg/kg*h for female rats. The model confirmed a biphasic decline of plasma radioactivity with a short first half life of elimination of ca. 3 hours which is probably mainly attributable to the elimination of the parent compound and a significantly longer second half life of ca. 53 hours probably related to the incorporation of radioactivity into biomolecules.
The corresponding concentration-time curves of the modelled and the measured data is shown in Figure 5.1.2-01 below.

Figure 5.1.2-01: Timecourse of radioactivity in the plasma following a single oral dose of 2 mg/kg [furanone-4-^{14}C] BYI 02960. (upper curve: male rats; lower curve: female rats)



C. Excretion

The major route of excretion was renal. In total, approx. 79% of the dose administered was detected in the urine of male rats, the major part of this (ca. 75%) was excreted within 24 hours. Faecal excretion accounted for ca. 16.6% of the given dose in males. Also the major part of faecal residues (ca. 15% of

the dose) was excreted within the first day after treatment. For female rats, a somewhat higher renal excretion was observed. Approx. 91% of the dose was found in urine (ca. 87% excreted within 24 hours) and approx. 10% in faeces (ca. 9% excreted on day 1). These results are in good accordance to the excretion behavior detected in all other rat studies. The cumulative excretion results in percent of the administered radioactivity are summarized in Table 5.1.2-04.

Table 5.1.2.04: Cumulative excretion of radioactive residues via urine and feces after oral administration of [furanone-4-^{14}C]BYI 02960 to male and female rats

Time [h]	% of dose	
Feces	**Male, 2 mg/kg**	**Female, 2 mg/kg**
24	14.76	9.45
48	16.40	10.29
72	16.50	10.33
96	16.54	10.35
120	16.56	10.36
144	16.57	10.37
168	16.59	10.38
Urine		
4	8.74	6.52
8	33.86	18.32
12	59.55	-
24	75.04	87.31
48	78.40	90.44
72	78.75	90.89
96	78.86	91.14
120	78.91	91.24
144	78.94	91.33
168	78.96	91.37
Total	**95.56**	**101.70**

D. Radioactive residues in organs and tissues at sacrifice

Approx. 0.5% of the dose was detected in the body of male rats at sacrifice 168 hours after oral administration; only a very minor amount of 0.02% was found in the GIT. Residual concentrations of radioactivity were in the range of 0.0025 to 0.0336 mg/kg. The lowest concentration was detected in the plasma and the highest value in the thyroid. But basically, levels for most organs and tissues were very similar and in the range of approx 0.005 to 0.01 mg/kg. For female rats, approx. 0.2% of the dose was still present in the body at sacrifice and only a negligible proportion of 0.01% in the GIT. Residual concentrations of radioactivity were in the range of 0.0012 to 0.0131 mg/kg. The lowest concentration was detected in the plasma and the highest value in the thyroid. As for males, levels for most organs and tissues were very similar and in the range of approx 0.002 to 0.005 mg/kg. For most organs and tissues the residues in males were 2 - 3 x higher as compared to females. This difference is also very likely caused by the higher rate of incorporation of radioactivity into biomolecules. The equivalent

concentrations of the residual radioactivity in organs and tissues at sacrifice are presented in Table 5.1.2.-05.

Table 5.1.2.05: Total radioactive residues in organs and tissues at sacrifice after oral administration of [furanone-4-^{14}C]BYI 02960 to male and female rats. Data are presented as equivalent concentration [mg a.s. equiv./kg]

	Male, 2 mg/kg bw	Female, 2 mg/kg bw
Blood cells	0.0083	0.0038
Plasma	0.0025	0.0012
Carcass	0.0079	0.0031
Heart	0.0065	0.0029
Brain	0.0072	0.0033
Kidneys	0.0104	0.0045
Liver	0.0128	0.0081
Testes	0.0059	---
Ovaries	---	0.0039
Uterus	---	0.0035
Adrenal gland	0.0200	0.0114
Harderian gland	0.0241	0.0091
Thyroid gland	0.0336	0.0131
Spleen	0.0081	0.0032
Lung	0.0075	0.0048
Eye	0.0053	0.0048
Skin	0.0111	0.0047
Bone femur	0.0085	0.0049
Fat perirenal	0.0218	0.0058
Muscle leg	0.0069	0.0023

E. Identification / characterisation and quantification of residues

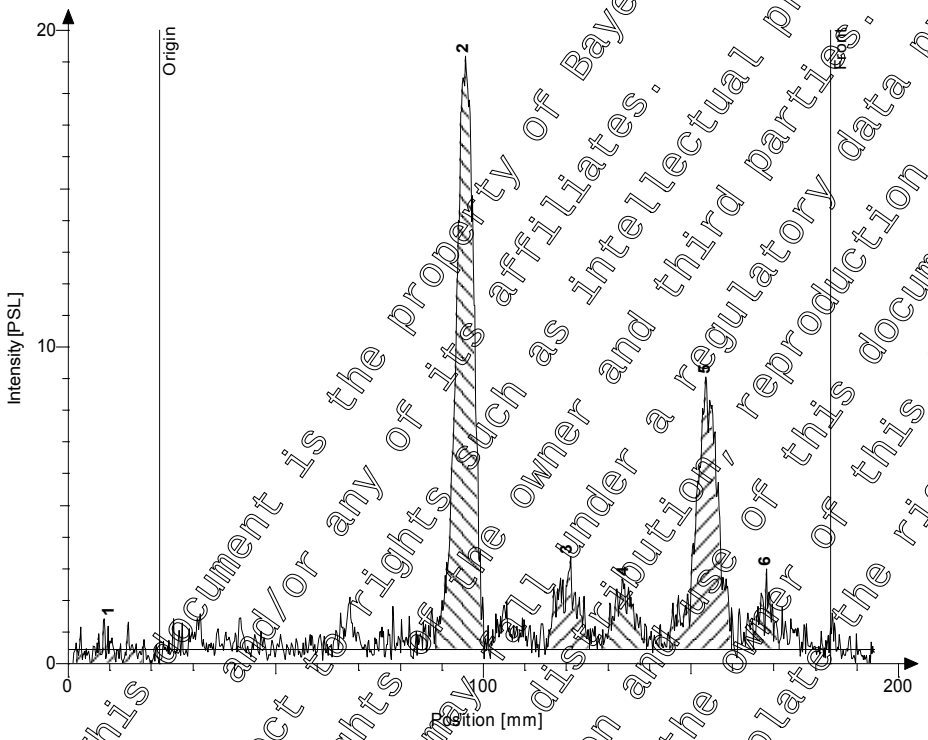
The following strategy was used for the identification of parent compound and metabolites:
The metabolic profile of the urine sample 12 - 24 h collected from male rats was compared with the profile of the urine pool 0 - 6 h of male rats from the organ metabolism study with [furanone-4-^{14}C]BYI 02960 for a first assignment of parent and metabolites. In this organ metabolism study (KIIA 5.1.2/03), all major compounds in urine had been identified by LC-MS/MS and/or HPLC co-chromatography and comparison.
The metabolite BYI 02960-difluoroethyl-amino-furanone was additionally identified by LC-MS/MS after isolation from urine. The trace metabolites BYI 02960-OH-SA and BYI 02960-iso-OH were identified by HPLC comparison with urine or faeces samples from the ADME study with the pyridinylmethyl label where these metabolites had been identified (KIIA 5.1.1/01). For all other samples, identification was achieved by co-chromatography and based on the comparison of retention times and fingerprints.

F. Metabolites in urine

The main constituent in urine was the unchanged parent compound representing ca. 50% of the dose in males and 70% in females. Only one major metabolite was detected, BYI 02960-OH, which accounted for ca. 14% of the dose in males and 11% in females. Other identified metabolites were BYI 02960-

difluoroethyl-amino-furanone, BYI 02960-OH-gluA (isomer 1 and 3), BYI 02960-desdifluoroethyl, and BYI 2960-OH-SA which accounted for less than 4% of the dose, each.
A very polar metabolite fraction (peak 1) represented 5.22% of the dose in the urine of male rats and 2.42% in female rats. This peak was isolated from a urine pool sample 0-48h of male rats and analyzed by TLC with radio detection. The sample could be separated into five compounds representing between 0.19% and 2.75% of the dose (see Figure 5.1.2-02), but none of these could be identified.

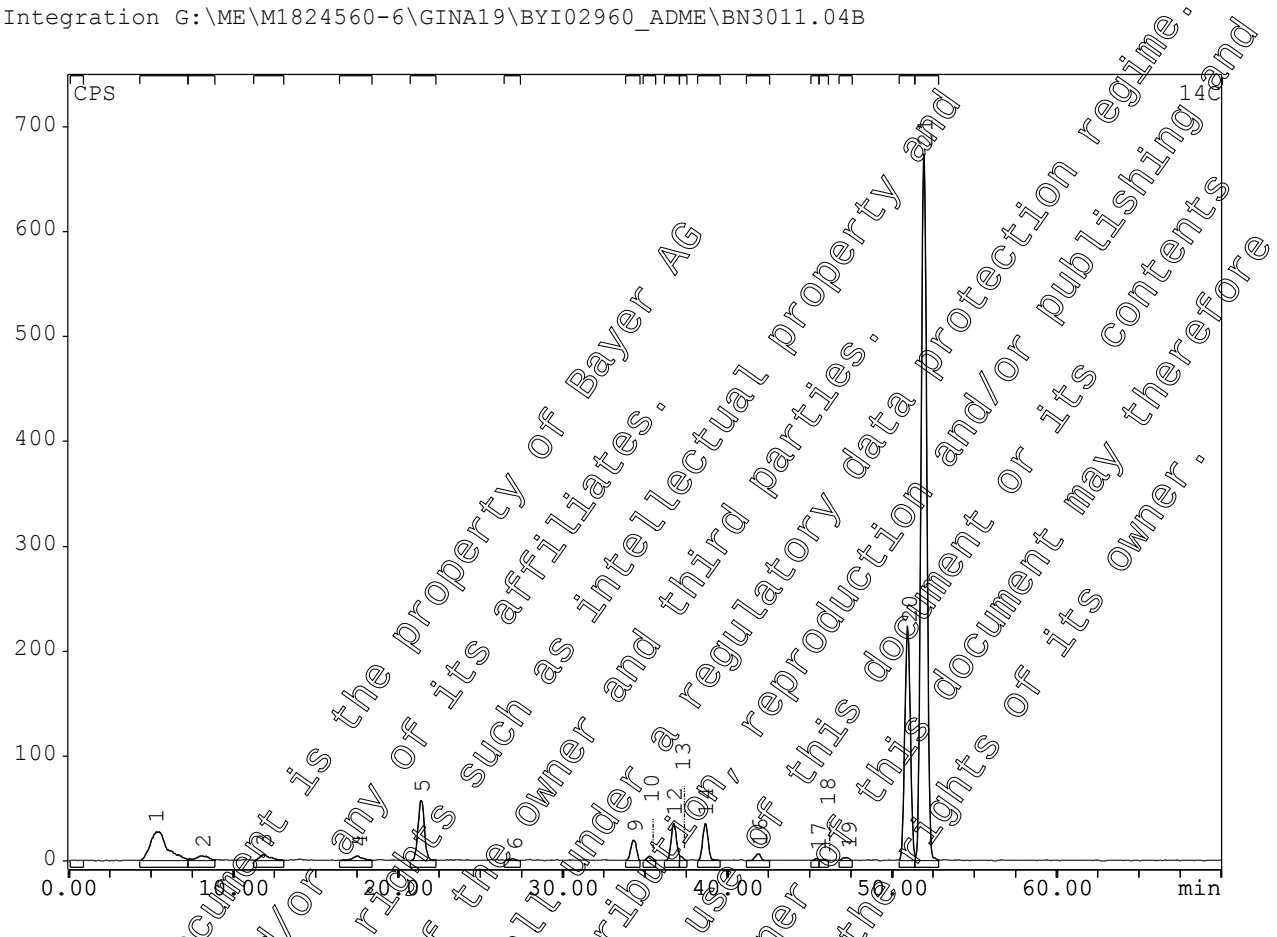
Figure 5.1.2.02:TLC sub-quantification of peak 1 isolated from urine 0 - 48 h of male rats after oral administration of 2 mg/kg [furanone-4-^{14}C]BYI 02960



In total, ca. 79% of the dose was detected in urine of male rats of which ca. 71% was identified and ca. 8% characterized. In female rats ca. 90% of the dose was excreted with urine of which ca. 86% was identified and ca. 4% characterized. With the exception of the polar metabolite (peak 1), all other characterized metabolites were found at levels below 1% of the given dose.

The results are summarized in Table 5.1.2-06. The peak numbers in the table correlate with those in the metabolic profile shown in Figure 5.1.2-03.
A representative chromatogram of the sampling interval 12 - 24 h is shown in Figure 5.1.2-03.

Figure 5.1.2.03:Metabolic profile of urine 12 - 24h of male rats after oral administration of 2 mg/kg [furanone-4-^{14}C]BYI 02960



F. Metabolites in feces

The parent compound in faeces of male rats represented ca. 6% of the total dose. The main constituent of the faecal residues in male rats was metabolite BYI 02960-OH which accounted for ca. 7% of the dose. In faeces of female rats, the parent compound was also detected at ca. 6% of the dose, while BYI 02960-OH made up ca. 3% of the dose.
Other identified metabolites were BYI 02960-difluoroethyl-amino-furanone, BYI 02960-desdifluoroethyl, BYI 2960-OH-SA, and BYI 2960-iso-OH which accounted for less than 0.3% of the dose, each.
In total, ca. 14.8% of the dose was detected in the faeces 0 - 24 h of male rats of which ca. 13.9% was identified and ca. 0.4% characterised. For female rats, ca. 9.5 of the dose was found in the faeces 0 - 24 h; ca. 9.0% was identified and ca. 0.1% characterised. All characterised metabolites were found at levels below 0.2% of the given dose. The results are summarized in Table 5.1.2.06.

G. Comparison of the metabolic profiles in urine and faeces

BYI 02960 was metabolised to approx. 20 metabolites. The parent compound represented the predominant part of the radioactivity in urine while in feces the amounts of the metabolite BYI 02960-OH were more or less similar. All other identified and characterised metabolites represented a minor part of the dose. The metabolic profiles in urines and faeces were very similar for both sexes but male

rats exhibited a higher rate of metabolite formation as compared to female animals. A summary of the distribution of the parent compound and metabolites in the excreta is provided in Table 5.1.2.06.

Table 5.1.2-06: Amounts of metabolites expressed as % of the total dose administered in the excreta of rats after administration of [furanone-4-^{14}C]BYI 02960

	Male: 2 mg/kg	Female: 2 mg/kg
Parent compound	54.68	75.96
difluoroethyl-amino-furanone	3.49	0.96
BYI 02960-OH-gluA (isomer 1)	1.13	0.49
BYI 02960-OH-gluA (isomer 3)	2.17	1.01
BYI 02960-des-difluoroethyl	2.12	3.35
BYI 02960-OH-SA	0.24	0.34
BYI 02960-OH	20.60	13.25
BYI 02960-iso-OH	0.26	0.07
Total identified	**84.70**	**95.42**
unknown 1*	5.30	2.45
unknown 2	0.46	0.19
unknown 3	0.57	0.05
unknown 4	0.03	---
unknown 5	0.40	0.12
unknown 6	0.05	0.16
unknown 7	0.02	0.09
unknown 8	0.03	0.11
unknown 9	0.24	0.44
unknown 10	0.02	0.02
unknown 11	0.25	0.23
unknown 12	---	0.14
unknown 13	0.53	0.02
unknown 14	0.10	0.06
unknown 15	0.02	---
Total characterized	**8.00**	**4.08**
Total	**95.55**	**101.75**

* unknown 1 from urine of male rats was further characterized by TLC analysis of the isolated metabolite fraction.

Five compounds were detected accounting for 0.19 - 2.75% of the dose administered

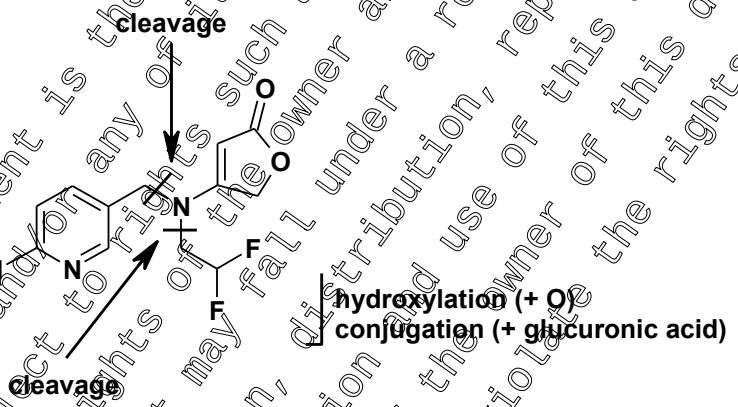
H. Biotransformation pathway

The principal metabolic reactions of [furanone-4-^{14}C]BYI 02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid or with sulfate,
- cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl,
- cleavage of the molecule at the pyridinylmethyl bridge forming BYI 02960-difluoroethyl-amino-furanone, and
- cleavage of molecule at the nitrogen-carbon bond next to the furanone moiety followed by further conversion to C1 and C2 compounds of the natural pool including complete degradation to carbon dioxide.

Figure 5.1.2-04 schematically shows the positions in the molecule, which are involved in these metabolic reactions.
The proposed biotransformation pathway of [furanone-4-^{14}C] BYI 02960 is presented in Figure 5.1.2-05.

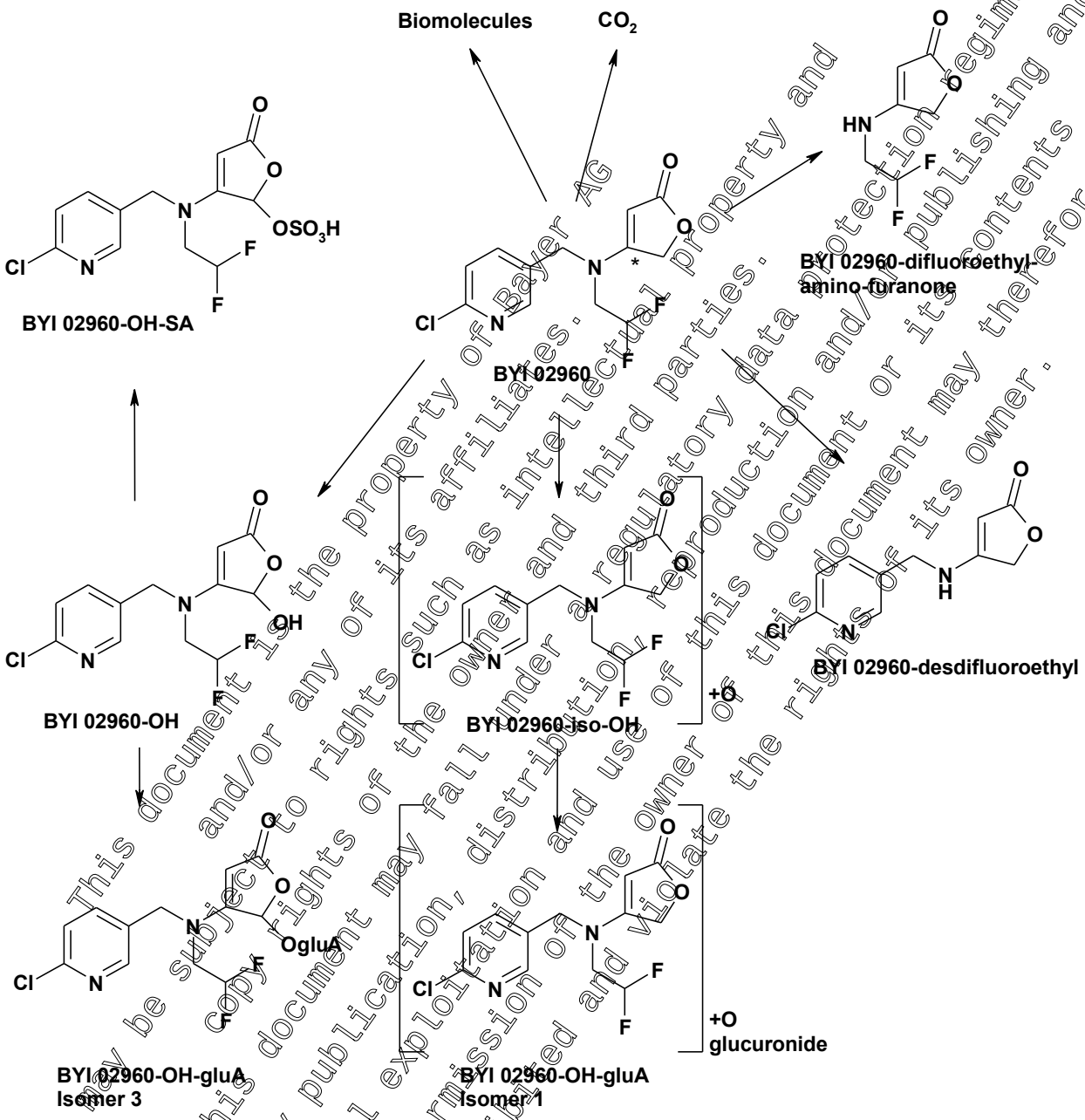
Figure 5.1.2-04: Positions involved in metabolic degradation of BYI 02960



III. Conclusions

- BYI 02960 was almost completely absorbed because >79% of the administered dose was detected in the urine and the body without GIT at sacrifice.
- The distribution of the radioactivity within the body was fast and the maximum plasma level (C_{max}) was reached within 1.5 hours after administration for both, male and female rats.
  The radioactivity level declined to ca. 50% of C_{max} after 8 hours and to <1% of C_{max} after 48 hours after dosing
- Excretion of radioactivity was fast and mainly renal. Female rats showed a slightly higher renal excretion rate of ca. 91% than males with ca. 78%.
- The major part of the dose (>89% for males and >96% for females) was excreted within 24 hours after treatment.
- Parent compound, one major and six minor metabolites were identified in urine and feces. Identification rates were >90% of the total radioactivity in urine and in feces.
- Approx. 85% of the dose was identified in excreta of male rats and approx. 95% in excreta of females. Another ca. 8% of the dose in males and ca. 4% in females was characterized by their chromatographic behavior.
- All metabolites representing >1% of the dose were identified with the exception of a very polar fraction which probably contained biomolecules formed by complete oxidative degradation of the furanone ring to C1 and C2 compounds.
- The metabolic transformation of BYI 02960 was principally oxidative and took place at least at 3 different structural positions of the test compound.
- Basically, male rats showed a higher rate of metabolism with only ca. 55% of unchanged parent compound found in excreta whereas 76% of unchanged BYI 02960 was found in the excreta of female rats. But the metabolic patterns were very similar in males and females.
- Male rats also showed a higher proportion of the polar metabolite fraction and higher residues in the body at the time of sacrifice as well as a higher proportion of radioactivity in exhaled air. These observations suggest that the furanone-4-^{14}C radiolabel is not completely stable and that a small part of the dose obviously underwent biotransformation to C1- and C2-fragments resulting in an incorporation of radioactivity into biomolecules, particularly in male rats.
- The metabolic pattern was in good accordance with that obtained from the corresponding organ metabolism rat study.

Figure 5.1.2-05: Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in rats



* position of ^{14}C label; gluA = glucuronic acid

Report:	KIIA 5.1.2/02, [redacted]; 2011
Title:	Quantitative whole body autoradiography of [furanone-4-^{14}C]BYI 02960 in male and female rats: distribution of total radioactivity and elimination from blood, organs and tissues after single oral administration including determination of radioactivity in the excreta and exhaled $^{14}CO_2$
Report No & Document No	MEF 11/275 M-409859-01-2
Guidelines:	OECD Guideline for Testing Chemicals, 417: Toxicokinetics US EPA Health Effects Test Guidelines OPPTS 870.7485 Metabolism and Pharmacokinetics PMRA Regulatory Directive Dir2005-01: Section 11: Toxicokinetic studies Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	Yes, fully compliant US EPA – FIFRA Good Laboratory Practice (40 CFR Part 160) Principles of Good Laboratory Practice – German Chemical Law (Chemikaliengesetz), dated 2002-06-20, current version of Annex 1 JAPAN MAFF - Notification on the Good Laboratory Practice Standards for Agricultural Chemicals (11 Nousan 6283) notified 1999-10-01
Testing Facility and Dates	[redacted] Experimental work: 2008-04-10 - 2010-11-18

Executive Summary

BYI 02960, labelled with ^{14}C in the 4-position of the furanone ring, was readily absorbed from the gastrointestinal tract and evenly distributed throughout the body immediately after administration. The excretion of radioactivity via urine and faeces was almost complete after 2 days with renal excretion as the predominant route. Until the end of the study at day seven, more than 80% of the dose was detected in the urine of both sexes. From 0.96% (females) to 3.25% (males) of the dose was exhaled as $^{14}CO_2$ during a sampling period of 48 hours. This demonstrated that for a small portion of the dose the furanone ring of the molecule obviously underwent extensive biotransformation to C1- and C2-fragments and the terminal product $^{14}CO_2$.

In male rats, maximum radioactivity concentrations ($C_{EQ_{max}}$ expressed as µg a.s. equiv. /g) were reached for almost all organs and tissues at one hour after administration (t_{max}). At this time, the values for liver, kidney, brown fat, myocardium, almost all glandular and hormonal organs, and the olfactory bulb were higher than in blood, suggesting a preferred clearance from blood and distribution to these organs which are mainly responsible for metabolism (liver), excretion (kidney) and secretion (*e.g.* adrenal gland, pancreas). A similar distribution - although on a lower level – was obtained at day seven. The relatively high radioactivity concentration in the nasal mucosa at this time is due to the exhalation of $^{14}CO_2$.

In female rats, maximum concentrations were also reached for all organs and tissues except the olfactory bulb at one hour after administration (t_{max}). The concentrations in liver, kidney, myocardium, almost all glandular and hormonal organs, and the olfactory bulb were higher than in blood at this time, suggesting again a preferred clearance from blood and distribution to those organs that are responsible for metabolism (liver), excretion (kidney) and secretion (*e.g.* adrenal gland, pancreas). A similar distribution - although on a rather low level - was obtained at day seven. As already shown for male

rats, the relatively high radioactivity concentration in the nasal mucosa is directly related to the exhalation of $^{14}CO_2$.
The concentrations declined following a biphasic kinetics. The second and slower decline phase, which started after 24 hours in males and after 48 hours in females, is probably linked to small carbon units (C1- or C2-fragments) that entered the carbon pool for the biosynthesis of endogenous compounds.
At the end of the study (day 7), low radioactive residues were measured in almost all organs and tissues due to the incorporation of C1- or C2-fragments into the endogenous carbon pool. The terminal residues were always higher by a factor of 1.4 to 4.7 in males as compared to females. A similar ratio of approx. 3 was also found for the formation of $^{14}CO_2$ between males and females. This is presumably due to quantitative differences in metabolism leading to more C1- and C2-fragments and also higher incorporation of these components into the endogenous carbon pool in male rats.
From the results of this study, any significant accumulation or retention of [furanone-4-^{14}C]BYI 02960 in male and female rats can be excluded.

I. Material and Methods

A. Material

1. Test Material:

IUPAC Name:	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name:	BYI 02960
Common name:	flupyradifurone
Empirical formula:	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass:	288.68 g/mol
Water solubility:	pH 4 = 3.2 mg/L, pH 7 = 3.2 mg/L pH 9 = 3.0 mg/L
n-Octanol/water partition coefficient:	pH 4 = 1.2, pH 7 = 1.2, pH 9 = 1.2
Labelling:	[furanone-4-^{14}C]
Specific radioactivity of the radiolabelled batches:	2.94 MBq/mg = 2.364 x 10^5 dpm/µg = 106.46 µCi/mg = 30.73 Ci/mol
Specific radioactivity used for administration:	3.94 MBq/mg = 2.364 x 10^5 dpm/µg = 106.46 µCi/mg = 30.73 Ci/mol
Radiochemical purity:	> 99 % (certified, HPLC and TLC with radiodetection)
Dose level:	5 mg/kg body weight
Vehicle:	0.5 % aqueous tragacanth suspension
Stability of the test material:	The stability of [pyridinylmethyl-^{14}C]BYI 02960 was determined by radio-HPLC of the administration suspensions of each test immediately after dosing

2. Test Animals:

Species:	Rat (*Rattus norvegicus domesticus*)	
Strain:	Wistar Hsd/Cpb: WU	
Breeding facility:	[redacted]	
Sex and numbers involved:	Males:	8 + 1 control animal
	Females:	8 + 1 control animal
	control animals were dosed with non radiolabelled test item	
Age:	7 weeks (male rats) and 8 weeks (female rats) at the time of delivery	
Body weight:	Males:	196 - 208 g at the time of administration
		200 - 208 g at the time of sacrifice
	Females:	192 - 204 g at the time of administration
		190 - 208 g at the time of sacrifice
Acclimatization:	Makrolon® cages on wood shavings in the test facility for about 7 days prior to the administration.	
Identification:	Cage cards on which the study number, test compound name and individual animal number were displayed. Additional labelling with water-insoluble spots on the tail	
Housing:	After administration of the radiolabelled test substance individually in Makrolon® metabolism cages under conventional hygienic conditions in air-conditioned rooms Temperature 22 - 24 °C, relative humidity 45 - 60 %. 12 / 12 hours light / dark cycle, air change 10 - 15 times per hour	
Feed and water:	Rat/mice maintenance long life diet (no. 3883.0.15), supplied by Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland (ca. 16 g per animal and day) Last feeding ca. 16 h prior to dosing Next feeding ca. 6 h after dosing Tap water from municipal water supply, *ad libitum*	

B. Study Design

1. Dosing

Each of the rats (8 per gender) orally received 5 mg/kg bw of [furanone-4-^{14}C]BYI 02960 suspended in 0.5 % aqueous tragacanth after ca. 16 h of fasting. A control animal of each sex was treated with 5 mg/kg of non-radiolabelled BYI 02960. The dosing suspensions (0.3 mg/mL) were prepared one day before dosing and stored below 4°C.

The suspensions were administered to the rats by oral gavage using a syringe attached to an animal-feeding knob cannula. Each animal received a volume of 2 mL (10 mL/kg bw calculated for a nominal body weight of 200 g). The amounts of the administered test substance were calculated by dividing the amount of the administered radioactivity by the specific radioactivity. The actual mean administered dose of [furanone-4-^{14}C]BYI 02960 was 5.25 mg/kg bw for male and 4.97 mg/kg bw for female rats. The stability of the test compound in the suspensions was assured by radio-HPLC analysis after administration.

2. Collection of excreta

After administration of the radiolabelled test compound, the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine, faeces, and expired air. Urine was collected separately for each animal in a cryogenic trap cooled with dry ice in intervals of 1h, 4 h, 8 h, 24 h, and every 24 h until 168 h. The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction. The radioactivity was determined by LSC.
The faeces samples were collected every 24 h separately for each animal before they were lyophilised, weighed, and homogenised. The radioactivity was determined by combustion/LSC.

3. Trapping of expired air

Carbon dioxide and other volatiles in the expired air were collected from four male and four female animals for the time ranges 0 - 24 h and 24 - 48 h. The respective metabolism cages were attached to a high velocity air pump and ventilated with ca. 2 L of air per minute and cage. The air was passed through a trapping system of two gas-washing bottles each containing about 150 - 200 mL of a 1:1-mixture of ethanolamine/ethanol. At sampling, the exact volume was determined, from which an aliquot was taken for the determination of radioactivity by LSC.

4. Sacrifice and preparation of carcass for autoradiography

One animal of each sex was sacrificed using carbon dioxide at 1, 4, 8, 24, 48, 72, 120, and 168 h post administration, respectively. The control animals were sacrificed 4 h after dosing.
After sacrifice, the animals were fixed in a stretched position using a metal template and immediately frozen at ca. -70 °C in a dichloromethane/dry ice bath. After removal of the template, the animal body together with a series of blood standards (^{14}C labelled reference compound in bovine blood) was embedded in a slurry of carboxymethylcellulose (7 to 8 %) on the platform of the cryomicrotome (Leica CM 3600, Leica Instruments GmbH, 69226 Nussloch/ Germany).

5. Autoradiography

Sagittal sections of 50 µm thicknesses were cut at ca. -25 °C using a cryomicrotome, attached to adhesive tape, and lyophilised overnight in the cooling cabinet of the microtome. Four to five sections exhibiting the relevant organs and tissues were prepared from each animal. All cryosections were exposed at approx. +4 °C in a lead shielding box in order to minimize the background signal for 6 to 120 hours before being scanned with the Fuji BAS 5000® image analyzer. The sections were stored at about -20 °C at all times except during exposure.
The sections of the control animals were exposed under identical conditions, using the longest exposure time which had been chosen for rat sections of animals treated with the radiolabelled compound. No blackening of typical animal structures could be observed after an exposure time of 120 hours for male and female animals.
The digital images of the radioluminograms allowed for the assessment of the radioactivity concentration distribution in different organs and tissues. The autoradiograms were quantitatively evaluated using the AIDA® software (Raytest, Straubenhard, Germany). Defined areas were set and integrated in each organ or tissue or substructure thereof. After background subtraction, a value of the photostimulated luminescence (PSL) per mm^2 was obtained, which is proportional to the equivalent concentration of the radioactivity in that particular tissue.

Two series of 8 calibration standards were prepared by spiking bovine control blood with different concentrations of a ^{14}C-radiolabelled reference compound. The concentrations covered a range from approx. 400 to 2,000,000 dpm/g. The radioactivity of each blood calibration standard was determined by combustion/LSC and the mean values of each standard were used to establish a calibration graph for the correlation of (PSL - Bkg)/ mm^2 to the radioactivity in dpm/g tissue by linear regression analysis. The obtained regression factors were used to calculate the concentration of the radioactivity in dpm/g in the lyophilised rat sections. These values were corrected for the self-absorption of the various types of tissues using self absorption factors published in the literature. To express the values as equivalent concentrations, the radioactivity concentrations given in dpm/g were divided by the specific radioactivity of the test substance in dpm/µg.

6. Radioactivity measurement

The measurement of radioactivity in liquid samples was carried out by liquid scintillation counting (LSC) of 1 - 3 replicates. All solid samples and blood standards were weighed and combusted in an oxygen atmosphere with an Oxidizer 307/387 (Packard Instruments).
For all samples, the limit of detection (LOD) was established at ca. 20 dpm measured per aliquot after correction for the background radioactivity. The limit of quantification (LOQ) for each individual measurement was established as 2 times of the background radioactivity (dpm) of each instrument/method. This background counting rate was in a range between 12 - 30 cpm (approximately 12 - 30 dpm) and it was automatically subtracted from the measuring results. A quench and counting efficiency correction for transformation of gross counts (cpm) into net counts (dpm) was automatically performed by the instruments. 1

II. Results and Discussion

A. Distribution

The distribution of BYI 02960 in male and female rats was investigated by quantitative whole body autoradiography (QWBA) using the radioluminography (RLG) technique. The data were obtained over a testing period of 7 days in male and female rats following a single oral administration of radiolabelled [furanone-4-^{14}C]BYI 02960 at a dose level of 5 mg/kg body weight. Eight male and female rats each were treated and sacrificed at 1, 4, 8, 24, 48, 72, 120, and 168 hours after dosing.
For almost all organs and tissues, maximum concentrations were reached at one hour after administration. At this time, the values for several organs, like liver, kidney, brown fat, myocardium, almost all glandular organs, and the olfactory bulb were higher than in blood. In case of female rats, the olfactory bulb reached its maximum concentration only after 8 hours. For the remaining organs and tissues, *i.e.* muscle, perirenal fat, brain, thymus, testis, and lung, lower values were calculated.
The radioactivity administered with [furanone-4-^{14}C]BYI 02960 was rapidly absorbed and distributed to the entire body, preferably to those organs or tissues that are responsible for metabolism (liver), excretion (kidney) and secretion *(e.g.* adrenal, thyroid, Harderian and salivary glands), and to the organs or tissues having contact to expired air (olfactory bulb and nasal mucosa). The lowest transfer was determined for nervous and fatty tissues.
The tissue/blood-concentration ratios at t_{max} were highest for the adrenal gland (factor 1.90), followed by liver (1.80), kidney (1.67), olfactory bulb (1.64), thyroid and Harderian glands (1.41), myocardium

(1.33), salivary gland (1.35) and pancreas (1.30). The relatively lowest values were calculated for the spinal cord (0.38), brain (0.34), and perirenal fat (0.15).
The equivalent concentrations in blood, organs and tissues declined following a biphasic kinetics showing a fast phase from 1 to 24 hours (females: 48 h) and a slower decline from 24 to 168 hours. After seven days the radioactivity concentrations in blood and all organs and tissues had declined significantly but were still higher in almost all of the organs than in blood. Slightly lower values were detected in the skeleton muscle, myocardium, lung, pancreas, pineal and vitreal body. Higher tissue/blood concentration ratios were in a range between 1.05 (perirenal fat, males), 1.31 (uterus) and 14.12 (nasal mucosa). All factors are average values of male and female rats.
The results are summarized in Table 5.1.2-07 (male rats) and Table 5.1.2-08 (female rats) below.

B. Excretion

The radioactivity administered with [furanone-4-^{14}C]BYI 02960 was excreted rapidly and completely within ca. 48 hours. The major part of the radioactivity (up to 81% in male and up to 88% in female rats) was excreted with urine and the minor part (minimum ca. 16% for male and ca. 7% for female rats) with faeces. Around two days after administration, the excretion was almost complete.
The exhalation of $^{14}CO_2$ was tested with male and female animals for a period of 48 hours. Between 2.02% and 3.25% (males) and 0.50% and 0.96% (females) of the administered radioactivity was exhaled during this period. This demonstrated that for a small portion of the dose (higher in males than in females) the furanone ring of the molecule obviously underwent biotransformation to C1- and C2-fragments and the terminal product $^{14}CO_2$. The excretion behavior is summarized in Table 5.1.2-09 (male rats) and Table 5.1.2-10 (female rats).

III. Conclusions

The insecticide BYI 02960 labelled with ^{14}C in the 4-position of the furanone ring, was readily absorbed from the gastrointestinal tract of male and female rats and evenly distributed throughout the body immediately after single oral administration. The radioactivity was rapidly cleared from the blood and distributed primarily to those organs or tissues that are responsible for metabolism, excretion and secretion.
The excretion of radioactivity via urine and faeces was almost complete two days after administration with more than 80% of the dose detected in the urine of both sexes. The detection of $^{14}CO_2$ in the exhaled air during a sampling period of 48 hours (up to 3.25% in male and 0.96% in female rats) demonstrated that for a small portion of the dose the furanone ring obviously underwent biotransformation to C1- and C2-fragments and the terminal product $^{14}CO_2$.
Peak concentrations of radioactivity for almost all organs and tissues were reached already one hour after dosing. From then onwards, the concentrations declined following a biphasic kinetics. The second slower decline phase, which started after 24 hours in males and after 48 hours in females, is probably due to the formation of small carbon units (C1- or C2-fragments) that entered the carbon pool used for the biosynthesis of endogenous compounds. This is presumably also the reason that at the end of the test (day 7) low radioactive residues were still measured in almost all organs and tissues.
The terminal residues were always by a factor of 1.4 to 4.7 higher in males than in females. A similar ratio of approx. 3 was also found for the formation of $^{14}CO_2$ in males as compared to females. The

reason is probably a gender specific quantitative difference in metabolism leading to more C1- and C2-fragments and also a higher incorporation of these components into the endogenous carbon pool.
From the results of this study, any significant accumulation or retention of [furanone-4-^{14}C]BYI 02960 in male and female rats can be excluded.

Table 5.1.2-07: Distribution of radioactivity in organs and tissues of male rats after a single oral dose of 5 mg [furanone-4-^{14}C]BYI 02960/kg bw

Organ or tissue	Equivalent concentration CEQ [µg a.s. equiv./g]							
	Time of sacrifice [hours after administration]							
	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h
Blood	3.896	2.737	1.634	0.086	0.039	0.039	0.024	0.017
Liver	6.860	4.734	2.976	0.303	0.168	0.150	0.080	0.036
Renal cortex	5.743	4.346	2.247	0.146	0.056	0.050	0.035	0.021
Renal medulla	7.529	6.423	3.037	0.205	0.071	0.075	0.042	0.027
Kidney total	6.636	5.384	2.642	0.176	0.063	0.067	0.038	0.024
Brown fat	4.286	2.554	1.665	0.145	0.110	0.083	0.059	0.030
Perirenal fat	0.552	0.418	0.258	0.033	0.020	0.012	0.031	0.018
Skeleton muscle	3.733	2.896	1.426	0.073	0.026	0.029	0.018	0.014
Myocardium	5.274	3.544	2.131	0.097	0.037	0.035	0.026	0.016
Lung	2.794	2.555	1.250	0.077	0.030	0.028	0.017	0.013
Spleen	3.754	2.878	1.464	0.103	0.056	0.057	0.035	0.019
Pancreas	4.960	3.044	2.003	0.117	0.044	0.046	0.026	0.016
Bone marrow	3.320	2.395	1.309	0.182	0.104	0.081	0.041	0.019
Testis	2.493	2.066	1.229	0.080	0.044	0.042	0.030	0.021
Brain	1.372	1.055	0.715	0.081	0.066	0.060	0.052	0.033
Spinal cord	1.624	1.253	0.788	0.108	0.080	0.079	0.067	0.042
Pituitary gland	4.639	2.896	1.766	0.123	0.077	0.073	0.040	0.026
Pineal body	3.763	2.534	1.506	0.105	---	0.048	0.031	0.014
Adrenal gland	7.487	4.793	2.914	0.358	0.143	0.131	0.072	0.039
Thymus	3.869	2.635	1.587	0.140	0.106	0.097	0.045	0.022
Thyroid gland	5.391	3.848	2.280	0.304	0.253	0.174	0.080	0.036
Salivary gland	5.224	3.691	2.237	0.138	0.063	0.059	0.037	0.020
Nasal mucosa	1.943	1.880	1.727	0.592	0.419	0.401	0.179	0.164
Vitreal body	1.785	1.875	1.301	0.197	0.091	0.059	0.049	0.015
Harderian gland	5.384	4.421	---	0.443	0.367	0.313	0.090	0.050
Olfactory bulb	5.823	4.097	2.875	0.547	0.434	0.371	0.128	0.052

--- : Organ or tissue was visible in the rat sections but not discernible in the radioluminograms

Table 5.1.2-08: Distribution of radioactivity in organs and tissues of female rats after a single oral dose of 5 mg [furanone-4-^{14}C]BYI 02960/kg bw

Organ or tissue	Equivalent concentration CEQ [µg a.s. equiv. /g]							
	Time of sacrifice [hours after administration]							
	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h
Blood	4.250	2.587	1.341	0.061	0.009	0.007	0.006	0.006
Liver	7.757	4.648	2.705	0.143	0.024	0.018	0.011	0.009
Renal cortex	5.937	3.373	2.027	0.094	0.015	0.010	0.008	0.008
Renal medulla	7.971	5.223	3.525	0.156	0.018	0.015	0.012	0.011
Kidney total	6.954	4.298	2.776	0.125	0.016	0.012	0.010	0.009
Brown fat	3.604	2.221	0.938	0.089	0.018	0.019	0.010	0.008
Perirenal fat	0.654	0.365	0.179	0.014	<LOQ	<LOQ	<LOQ	<LOQ
Skeleton muscle	4.498	2.441	1.398	0.058	0.006	<LOQ	<LOQ	<LOQ
Myocardium	5.606	3.174	1.826	0.075	0.009	0.006	0.006	0.006
Lung	3.714	1.997	1.036	0.050	0.006	0.005	0.004	<LOQ
Spleen	4.238	2.273	1.313	0.068	0.013	0.010	0.007	0.007
Pancreas	5.546	3.081	1.691	0.082	0.010	0.006	0.005	---
Bone marrow	3.187	2.295	1.002	0.075	0.019	0.013	0.008	0.006
Ovary	4.143	2.246	1.447	0.075	0.012	0.011	0.006	<LOQ
Uterus	4.305	2.670	1.414	0.068	0.018	0.013	0.010	0.008
Brain	1.376	0.811	0.462	0.023	0.007	0.007	0.006	0.009
Spinal cord	1.464	0.941	0.495	0.029	0.009	0.009	0.008	0.011
Pituitary gland	4.838	2.950	1.516	0.076	0.012	---	---	---
Pineal body	3.176	2.444	1.244	0.053	---	---	---	---
Adrenal gland	7.927	4.739	2.498	0.172	0.057	0.036	0.021	0.026
Thymus	4.287	2.577	1.319	0.072	0.021	0.019	0.010	0.007
Thyroid gland	6.024	3.585	1.806	0.127	0.050	0.032	0.026	0.026
Salivary gland	5.732	3.450	1.833	0.085	0.013	0.010	0.007	0.006
Nasal mucosa	1.689	1.622	0.751	0.390	0.250	0.203	0.160	0.117
Vitreal body	1.831	1.615	1.190	0.212	0.041	0.032	0.013	0.009
Harderian gland	6.238	3.491	2.034	0.120	0.021	0.011	0.014	---
Olfactory bulb	---	---	2.393	0.194	0.068	0.064	0.021	0.017

--- : Organ or tissue was visible in the rat sections but not discernible in the radioluminograms

<LOQ: below limit of quantitation

Table 5.1.2-09: Excretion of radioactivity in urine, faeces and expired air of male rats after a single oral administration of 5 mg [furanone-4-^{14}C]BYI 02960/kg bw

	Percent of radioactive dose administered							
	Time of sacrifice [h after administration]							
	1	**4**	**8**	**24*)**	**48**	**72**	**120**	**168**
Exhaled air								
24 h					2.33	2.74	2.54	1.71
48 h					2.83	3.25	3.01	2.02
Urine								
1 h	4.30							
4 h		13.29	4.49	6.27	3.44	3.56	6.76	19.80
8 h			41.15	41.14	27.44	28.12	48.81	50.47
24 h				45.15	73.40	69.05	74.38	77.72
48 h					75.07	71.95	77.50	80.11
72 h						72.26	77.87	80.39
96 h							78.00	80.48
120 h							78.09	80.52
144 h								80.56
168 h								80.59
Faeces								
24 h	*	*	*	41.20	13.54	13.41	10.80	12.76
48 h					15.09	15.48	13.07	13.68
72 h						15.84	13.30	13.76
96 h							13.36	13.79
120 h							13.39	13.80
144 h								13.82
168 h								13.83
Sum total	**4.30**	**13.29**	**41.15**	**86.35**	**93.69**	**91.35**	**94.50**	**96.44**

*) : Because of the unypical excretion behavior (41.20% in faeces after 24 hours), this animal was not considered

* : faeces not collected

Table 5.1.2-10: Excretion of radioactivity in urine, faeces and expired air of female rats after a single oral administration of 5 mg [furanone-4-^{14}C]BYI 02960/kg bw

	Percent of radioactive dose administered							
	Time of sacrifice [h after administration]							
	1	**4**	**8**	**24**	**48 *)**	**72**	**120**	**168**
Exhaled air								
24 h					0.50	0.63	0.66	0.82
48 h					0.58	0.71	0.77	0.96
Urine								
1 h	3.82							
4 h		32.45	35.72	38.42	36.43	42.56	28.90	35.24
8 h			60.08	54.58	53.23	48.11	35.08	44.17
24 h				87.30	54.81	90.78	76.99	84.22
48 h					57.56	92.71	80.60	86.19
72 h						92.89	81.22	86.49
96 h							81.60	86.66
120 h							82.01	87.88
144 h								87.98
168 h								88.04
Faeces								
24 h	*	*	*	6.57	33.30	4.32	4.94	4.69
48 h					34.84	5.18	6.46	5.60
72 h						5.22	6.58	5.75
96 h							6.73	5.79
120 h							6.76	5.81
144 h								5.83
168 h								5.84
Sum total	**3.82**	**32.45**	**60.08**	**93.87**	**92.98**	**98.82**	**89.54**	**94.84**

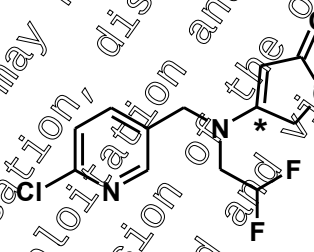
*) : Because of the unypical excretion behavior (41.20% in faeces after 24 hours), this animal was not considered

* : faeces not collected

Report:	**KIIA 5.1.2/03, [redacted]; 2011**
	[Furanone-4-^{14}C]BYI 02960 – Metabolism in Organs and Tissues of Male and Female Rats
Report No & Document No	MEF 11/271 M-414034-02-2
Guidelines:	OECD Guideline for Testing Chemicals, 417: Toxicokinetics US EPA Health Effects Test Guidelines OPPTS 870.7485, Metabolism and Pharmacokinetics Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	Yes, fully compliant US EPA – FIFRA Good Laboratory Practice (40 CFR Part 160) Principles of Good Laboratory Practice - German Chemical Law (Chemikaliengesetz), dated 2002-06-20, current version of Annex 1 JAPAN MAFF - Notification on the Good Laboratory Practice Standards for Agricultural Chemicals (11 Nousan 6283) notified 1999-10-01
Testing Facility and Dates	[redacted] Experimental work: 2010-10-2 - 2011-07-13

Executive Summary

The depletion of radioactive residues from plasma as well as organs and tissues, the excretion with urine and metabolism of the new insecticide BYI 02960 (common name: flupyradifurone) were investigated in male and female Wistar rats. Four male and four female rats were administered orally by gavage with a single dose of BYI 02960 in 0.5% aqueous Tragacanth® at a dose level of 3 mg/kg. The test compound was labelled with ^{14}C in the C-4-position of the furanone ring as shown below:



*^{14}C-label position

The animals were sacrificed 6 h after dosing. The total radioactivity was determined in urine for the time period 0 - 6 h as well as in plasma, liver, kidney, muscle (leg) and fat (perirenal) at sacrifice. The metabolism was investigated in urine and plasma, as well as in extracts of liver, kidney, muscle, and fat. The mean recovery for male rats was 100.60% and for female rats 98.09% of the given dose. The entire balances for the total radioactivity detected in urine, the combined GIT and faeces sample, skin, organs and tissues at sacrifice are shown in Table 5.1.2-11 below:

Table 5.1.2-11: Recovery of radioactivity in urine, plasma and organs 6 hours after a single oral dose of 3 mg/kg [furanone-4-^{14}C]BYI 02960

	Percent of given dose (mean values)	
	Male	**Female**
Urine	36.64	42.82
Plasma	0.70	0.55
Carcass	24.00	26.73
Kidneys	0.73	1.04
Liver	3.56	3.61
GIT+faeces	23.63	12.57
Skin	9.18	9.42
Fat (perirenal)	0.06	0.09
Muscle (leg)	2.09	1.26
Balance	**100.60**	**98.09**

For the 0 - 6 h collection period, the renal excretion in female rats was by a factor of approx. 1.17 higher than in male rats.
The highest radioactivity concentrations were detected in the liver (approx. 2.9 mg/kg for both sexes) and kidney (approx. 2.7 mg/kg for males and 4.3 mg/kg for females) as the main metabolic and excretory organs. The residue concentrations for plasma and the other tissues were comparable for both sexes and ranged from approx. 0.6 mg/kg for the perirenal fat to approx. 1.5 mg/kg for the leg muscle (see Table 5.1.2-12).

Table 5.1.2.12: Equivalent concentration of radioactivity in urine, plasma and organs 6 hours after a single oral dose of 3 mg/kg [furanone-4-^{14}C]BYI 02960

	Equivalent concentration [mg a.s. equiv./kg]	
	Male	**Female**
Plasma	1.316	1.385
Kidneys	2.732	4.346
Liver	2.928	2.937
Skin	1.141	1.281
Fat (perirenal)	0.558	0.651
Muscle (leg)	1.382	1.489

For the investigation of metabolism the pooled urine and plasma samples were analysed without further purification or extraction. Liver, kidney, muscle and fat samples were extracted using conventional methods. The extraction yield ranged from 95% to more than 99% of the total radioactivity.
Six hours after administration BYI 02960 was moderately metabolized. Metabolic reactions took place at least at 3 different positions of the molecule. The majority of components were identified (ca. 89 - 100% of the radioactivity in plasma and in extracts of organs and tissues as well as ≥ 88% of the radioactivity in urine).
In all samples of plasma, organs and tissues BYI 02960 was by far the largest component accounting for more than 72% of the total radioactivity. No metabolite accounted for more than 12% of the total

radioactivity. Also in urine the parent compound was the largest radioactive component (approx. 22% of the dose in males and 38% in females).
The metabolism was qualitatively similar in male and female rats. However, there were quantitative differences because the degradation of the parent compound was significantly higher in male as compared to female rats.

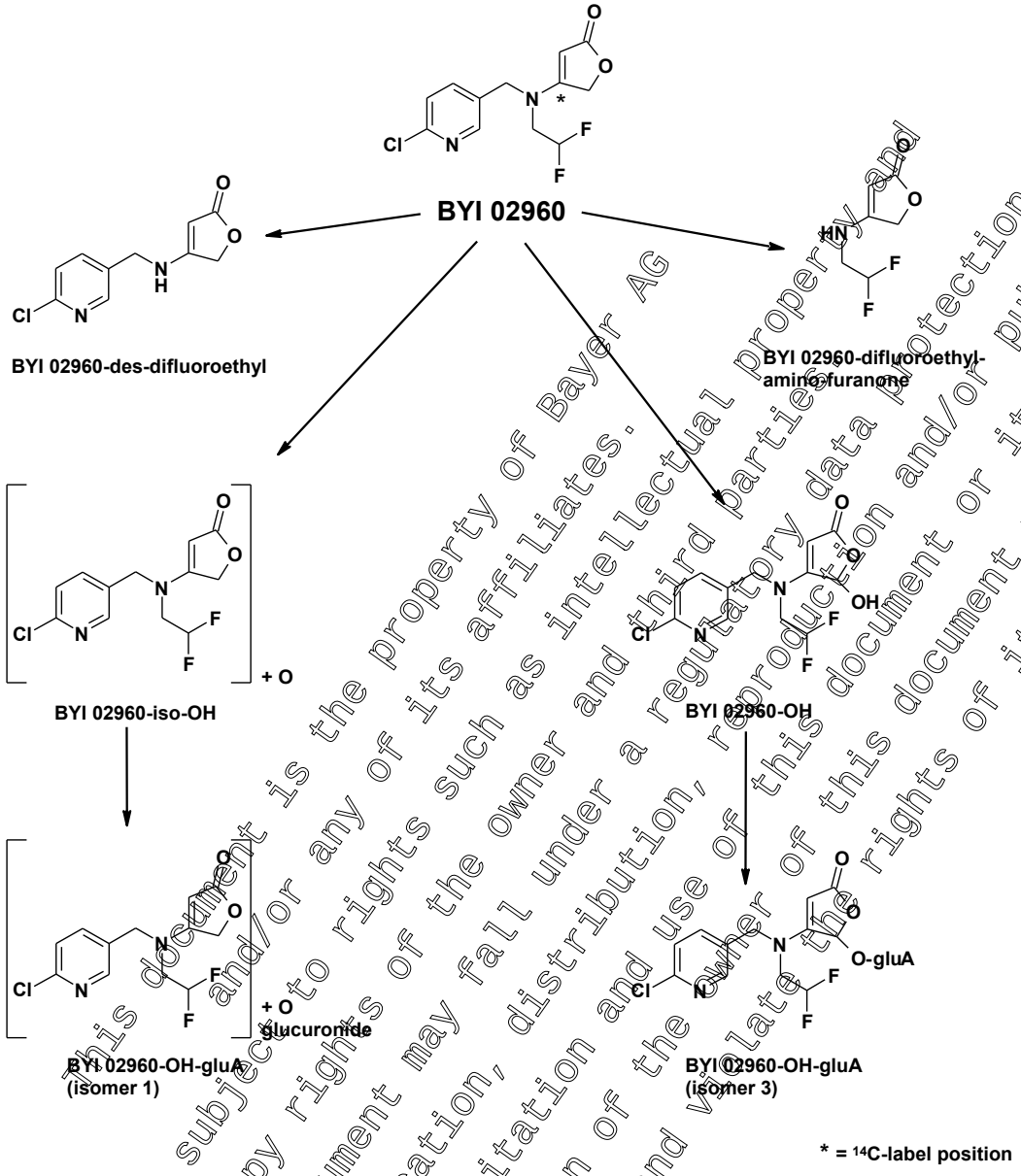
The principal metabolic reactions of [furanone-4-^{14}C]BYI 02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid,
- cleavage of the difluoroethyl group leading to BYI 02960-des-difluoroethyl and
- cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

The metabolism results are in good accordance with those obtained from the ADME rat study which was conducted with the [furanone-4-^{14}C] radiolabelled test compound (KIIA 5.1.2/01).

The proposed metabolic pathway is shown in Figure 5.1.2-06:

Figure 5.1.2-06: Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in rats



I. Material and Methods

A. Material

1. Test Material:

IUPAC Name:	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name:	BYI 02960
Common name:	Flupyradifurone
Empirical formula:	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass:	288.68 g/mol
Water solubility:	pH 4 = 3.2 mg/L, pH 7 = 3.2 mg/L pH 9 = 3.0 mg/L
n-Octanol/water partition coefficient:	pH 4 = 1.2, pH 7 = 1.2, pH 9 = 1.2
Labelling:	[furanone-4-^{14}C]
Specific radioactivity of the radiolabelled batches:	3.94 MBq/mg = 2.364 x 10^5 dpm/µg = 106.46 µCi/mg = 30.73 Ci/mol
Specific radioactivity used for administration:	3.94 MBq/mg = 2.364 x 10^5 dpm/µg = 106.46 µCi/mg = 30.73 Ci/mol
Radiochemical purity:	> 99 % (certified, HPLC with radiodetection) > 99 % (TLC with radiodetection)
Dose level:	3 mg/kg body weight
Vehicle:	0.5 % aqueous tragacanth suspension
Stability of the test material:	The stability of [furanone-4-^{14}C]BYI 02960 was determined by radio-HPLC of the administration suspensions immediately after dosing

2. Test Animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	Wistar Hsd/Cpb: WU
Breeding facility:	[redacted]
Sex and numbers involved:	4 male and 4 female animals
Age:	Males: ca. 6 weeks at the time of delivery Females: ca. 8 weeks at the time of delivery
Body weight:	194 - 213 g at the time of administration
Acclimatization:	Makrolon® cages on wood shavings in the test facility for 7 days prior to the administration
Identification:	Cage cards on which the study number, test compound name and individual animal number were displayed. Additional labelling with water-insoluble spots on the tail

Housing:	After administration of the radiolabelled test compound individually in Makrolon® metabolism cages under conventional hygienic conditions in air-conditioned rooms; Temperature 22 - 24 °C, relative humidity 47 - 71 % 12/12 hours light / dark cycle, air change 10 - 15 times per hour
Feed and water:	Rat/mice maintenance long life diet (no. 3883.0.15), supplied by Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland (ca. 16 g per animal and day) Last feeding ca. 16 h prior to dosing Next feeding ca. 6 h after dosing Tap water from municipal water supply, *ad libitum*

B. Study Design

1. Dosing

Each rat orally received 3 mg/kg bw of [furanone-4-^{14}C]BYI 02960 suspended in 0.5 % aqueous tragacanth after ca. 16 h of fasting. The dosing suspension (0.3 mg/mL) was prepared in a cold room at 5°C.

The suspension was administered by oral gavage using a syringe attached to an animal-feeding knob cannula. Each animal received a volume of 2 mL (10 mL/kg bw calculated for a nominal body weight of 200 g). The individual amounts of the administered test substance were calculated by dividing the amount of the administered radioactivity by the specific radioactivity. The actual mean administered dose of [furanone-4-^{14}C]BYI 02960 was 2.87 mg/kg bw for male rats and 3.03 mg/kg bw for female rats. The stability of the test compound in the suspension was assured by radio-HPLC analysis after administration.

2. Collection of excreta

After administration the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine and faeces. Urine was collected separately for each animal in a cryogenic trap cooled with dry ice from 0 - 6 h. The funnels for urine collection were rinsed with demineralised water at the end of the sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction. The radioactivity was determined by LSC. Faeces were collected from 0 - 6 h after dosing separately for each animal in a cryogenic trap cooled with dry ice. The radioactivity of the individual faeces samples was not determined, because they were added at sacrifice to the gastrointestinal tract (GIT) of the corresponding rat.

3. Plasma, organs and tissues at sacrifice

At sacrifice (6 h), the individual blood samples were collected in heparinized test tubes and separated into plasma and blood cells by centrifugation. After weighing, aliquots were taken for determination of radioactivity by LSC.

The dissected tissue samples (GIT including faeces, skin, and carcass including sedimented blood cells) were transferred into plastic vessels for recording of their individual fresh weights. The combined GIT/faeces-sample and an aliquot of depilated skin were lyophilized. After weighing and homogenization, aliquots were taken for determination of total radioactivity by combustion/LSC.

The whole carcass and blood cell samples were passed several times through a mincing machine in half-frozen state. From this tissue pulp, an aliquot was lyophilized, homogenized and weighed, before aliquots were taken for determination of the radioactivity by combustion/LSC.
Liver, kidneys, muscle and fat were weighed separately after collection. In order to obtain sufficient sample material for extraction of radioactive residues and metabolic profiling, the total radioactivity of the individual organs and tissue samples was not determined. Instead, respective sample pools were generated for each test group. The mean dpm/g-values which were used for further calculations were derived from the sum of extracts and solids of the respective samples after extraction.

4. Sacrifice

The rats were sacrificed in Pentobarbital-Na anaesthesia (Narcoren®, supplied [redacted] Germany) by transection of the cervical vessels and exsanguinated.

5. Measurement of radioactivity

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). All solid samples were combusted in an oxygen atmosphere using an oxidizer. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

6. Analytical methods

Samples were analyzed by radiochromatographic (HPLC) and spectroscopic (LC-MS, ^{1}H-NMR) methods.

7. High Performance Liquid Chromatography (HPLC)

HPLC-radioactivity analysis was performed on an Agilent 1100 system with radiometric- and UV-detection. The separation was carried out on a reversed phase column using a neutral water / acetonitrile gradient. Detailed information of the HPLC-methods and instruments set-up can be found in the report. All solvents were of HPLC-quality.
The chromatograms were recorded and quantitatively evaluated using the software package GINA® (raytest, Benzstraße 4, D-75334 Straubenhardt, Germany). The ^{14}C-trace of a chromatogram was divided into regions of interest (ROI's) according to the separated radioactive peaks. Additionally, background regions (BKG's) were defined and used for background correction. The background corrected area counts from all regions of interest were used for calculation of the quantitative distribution of the individual components in the sample. A quantifiable radioactive peak was regarded relevant if resulting in a signal approx. 2.5 times above the background noise.
For HPLC co-chromatography, an aliquot of the sample was mixed with the reference compound before injection. The detection was carried out either by UV-absorbance of the non-radiolabelled or by ^{14}C-detection of the radiolabelled reference compound. The time delay between the radioactivity and UV-absorbance detectors was compensated by a parameter set in the software. Chromatographic correspondence with the non-radiolabelled reference compound was assessed by comparison of the UV-trace and the associated ^{14}C-trace. Matching with the radiolabelled reference compound was assessed by comparison of the ^{14}C-chromatogram of the mixture with the ^{14}C-chromatogram of the sample without the reference compound.

8. Mass spectroscopy

Electro-spray ionization MS spectra (ESI) were obtained either with a TSQ 7000 instrument or with a LTQ Orbitrap XL mass spectrometer (Thermo or Finnigan, respectively, San Jose, CA, U.S.A.). The HPLC instrument used for chromatography was an Agilent HP1100 (Agilent, Waldbronn, Germany). The eluate of the HPLC-column was split between the UV-detector followed by a radioactivity detector (Ramona Star, Raytest, Straubenhardt, Germany) and the MS spectrometer.

9. ^{1}H-NMR spectroscopy

600 MHz ^{1}H-NMR-spectra were recorded using a BRUKER AV 600 instrument (Bruker, Karlsruhe, Germany).

10. Preparation of samples, extraction and analysis

Urine

The urine samples from all four animals of each test were combined. The amount of radioactivity applied for analysis was calculated from the in-life data of the respective samples. Aliquots thereof were taken without further sample processing for metabolite profiling by HPLC.

Plasma

The plasma samples from all four animals of each test were combined. The amount of radioactivity applied for analysis was calculated from the in-life data of the respective samples. Aliquots thereof were taken without further sample processing for metabolite profiling by HPLC.

Liver, kidney and muscle

The organ samples from all four animals of each test were combined for extraction. Consecutive solvent extractions were performed by macerating the samples 3-times with ACN/water (8/2, v/v) using a Polytron (model PT 3100) homogenization (ca. 3 min. at ca. 9,000 rpm). At each step, the respective sample was separated by filtration into extract and solids. The volume of each extract was measured and the radioactivity of an aliquot was determined by LSC. Aliquots were taken from the remaining final solids for radioactivity measurement by combustion/LSC. All extracts for which the radioactivity count of the respective aliquot was higher than 20 dpm were combined.
A Phenomenex Strata C18E SPE-cartridge was conditioned successively with an equal volume of methanol, water and ACN/water (8/2, v/v) before use. The combined organ extract was sucked slowly through the cartridge to remove the lipid fraction of the matrix. Unbound material was removed by washing the column with ACN/water (8/2, v/v). The percolate and rinse samples were combined, the volume was determined and aliquots were taken for radioactivity measurement. Possible bound material was removed by washing the column finally with DCM/MeOH (1/1, v/v). After determination of the volume, aliquots from each sample were taken for radioactivity measurement. The percolate and rinse samples were concentrated after addition of an emulsifier (200 - 500 µL). The pH-value was adjusted to approx. pH 7. Aliquots of this solution were taken for radioactivity measurement and metabolite profiling by HPLC.

Fat

The fat samples from all four animals of each test were combined for extraction. Consecutive solvent extractions were performed by macerating the sample at first once with a 1/1-mixture of n-heptane and ACN/water (8/2, v/v) and afterwards twice with ACN/water (8/2, v/v) using a Polytron (model PT

3100) homogenization (ca. 3 min. at ca. 16,000 rpm). At each step, the respective sample was separated by filtration into extract and solids. The filtrate from the first extraction step was transferred to a separatory funnel in order to separate the n-heptane from the ACN/water phase. The volume of each extract/phase was measured and the radioactivity of an aliquot was determined. Aliquots from the remaining final solids were taken for radioactivity measurement by combustion/LSC. All ACN/water-extracts for which the radioactivity count of the respective aliquot was above 20 dpm were combined. A Phenomenex Strata C18E SPE-cartridge was conditioned successively with an equal volume of methanol, water and ACN/water (8/2, v/v) before use. The combined fat extract was sucked slowly through the cartridge to remove the lipid fraction of the matrix. Unbound material was removed by washing the column with ACN/water (8/2, v/v). The percolate and rinse samples were combined, the volume was determined and aliquots were taken for radioactivity measurement. Possible bound material was removed by washing the column finally with DCM/MeOH (1/1, v/v). Following determination of the volume, aliquots from each sample were taken for radioactivity measurement. The percolate and rinse samples were concentrated after addition of an emulsifier (200 - 500 µL). The pH-value was adjusted to approx. pH 7. Aliquots of this solution were taken for radioactivity measurement and metabolite profiling HPLC.

11. Identification / Characterization and quantification of residues

The identification of parent compound and metabolites was mainly based on high resolution LC-MS analysis, HPLC co-chromatography using authentic reference compounds and chromatographic comparison. All chromatograms were integrated for quantification. Details of the procedure for identification, characterization and quantification of residues are provided in the report.

II. Results and Discussion

A. Recovery and renal excretion

The detailed recovery rates of radioactivity in urine, organs and tissues, skin and the combined GIT and faeces sample are shown in Table 5.1.2.13. The mean recovery for male rats was 100.60% and for female rats 98.09% of the given dose.

Urine was collected for the entire test period of 6 hours. The renal excretion of the administered radioactivity was 36.64% in male and 42.82% of the dose in female rats (Table 5.1.2-13). These results are in very good accordance to the urinary excretion behaviour of all other rat studies.

Table 5.1.2-13: Recovery of radioactivity in urine, plasma and organs 6 h after a single oral dose of 3 mg/kg BYI 02960

Percent of given dose (mean values)		
	Male	**Female**
Urine	36.64	42.82
Plasma	0.70	0.55
Carcass	24.00	26.73
Kidneys	0.73	1.04
Liver	3.56	3.61
GIT + faeces	23.63	12.57
Skin	9.18	9.42
Fat (perirenal)	0.06	0.09
Muscle (leg)	2.09	1.26
Balance	**100.60**	**98.09**

B. Radioactive Residues in Plasma and in Organs and Tissues

In male and female rats, approx. 40% of the dose was detected in organs and tissues, the highest amount was found in the residual carcass (approx. 24 - 27%), followed by skin, liver, muscle, kidney, and fat for which the lowest values were measured (approx. 0.06 - 0.09%).
The highest concentrations of radioactivity were detected in the liver (approx. 2.9 mg/kg for both sexes) and kidney (approx. 2.7 mg/kg for males and 4.3 mg/kg for females). The residue values for plasma and the other tissues were comparable for both sexes and ranged from approx. 0.6 mg/kg for fat up to approx. 1.5 mg/kg for muscle (see also Table 5.1.2-12 in the executive summary section).

C. Extraction Efficiency of Residues

Sample pools of liver, kidney, muscle, and fat were extracted using conventional methods. The resulting extracts represented between approx. 95% and more than 99% of the total radioactivity after purification using reversed-phase SPE. A summary of the extraction efficiency is shown in Table 5.1.2-14.

Table 5.1.2.14: Extraction efficiency (% of TRR) of liver, kidney, muscle and fat samples of male and female rats sacrificed 6 h after single oral administration of 3 mg/kg BYI 02960

	Liver	**Kidney**	**Muscle**	**Fat**
Male	94.9	97.8	99.3	96.2 + 0.9 *
Female	99.0	99.6	99.9	99.9

Heptane-phase

D. Identification / Characterization and Quantification of Residues

The following strategy was used for identification of parent compound and metabolites:
Spectroscopic investigations (LC-MS) were conducted on the 0 - 6 h urine samples of male rats. An HPLC co-chromatography of the 0 - 6 h urine sample of male rats with the radiolabelled reference compounds BYI 02960-difluoroethyl-amino-furanone, the isomers 1 and 3 of BYI 02960-OH-gluA and

BYI 02960-des-difluoroethyl was carried out. Furthermore, a comparison of the HPLC-radiochromatograms from all samples of each individual test was made.
Other peaks or peak groups additionally detected in the HPLC-profiles were designated as "unknown". They were characterized by their behavior during extraction and clean-up and the retention times in the HPLC-chromatograms. Parent compound and metabolites were quantified in the original urine and plasma samples and in the conventional acetonitrile/water extracts of organs and tissues by integration of the ^{14}C-signals in the HPLC-chromatograms.

1. Metabolites in urine

The dominating component in the urine was identified as parent compound (approx. 22.1% of the dose in males and 37.6% in females). The most prominent metabolite with more than 2% but less than 10% of the dose was identified as BYI 02960-OH. No other metabolites were identified in amounts above 2% of the dose. The identification rates amounted to approx. 89% of radioactivity for males and 98% for females. The detailed results of the metabolic profiles of the urine samples from all tests are summarized in Table 5.1.2-15.

Table 5.1.2-15: Quantification of parent compound and metabolites in urine of male and female rats sacrificed 6 h after single oral administration of 3 mg/kg [furanone-4-^{14}C]BYI 02960. Data are presented as % of the given dose

	Male	Female
Sampling period [h]	**0 - 6 h**	**0 - 6 h**
Parent compound	22.1	37.6
unknown	1.4	0.4
unknown	1.5	0.4
unknown	0.2	---
unknown	0.4	---
unknown	0.3	---
BYI 02960-difluoroethyl-amino-furanone	1.5	0.2
BYI 02960-OH-gluA (isomer 1)	0.4	---
unknown	0.2	---
BYI 02960-OH-gluA (isomer 3)	0.9	---
BYI 02960-des-difluoroethyl	0.8	0.8
unknown	0.2	---
unknown	0.1	---
BYI 02960-OH	6.9	3.3
Total identified	32.5	42.0
Total characterized *	4.1	0.8
Sum total	**36.6**	**42.8**
Identification rate	**88.8%**	**98.1%**

* : Peaks were characterized based on their retention time in HPLC-analysis

2. Metabolites in plasma

In the plasma of female rats, only the parent compound was detected. In the plasma of male rats, the parent compound was the dominating component (approx. 1.09 mg/kg). Three minor metabolites with

residue-concentrations below 0.11 mg/kg were detected two of them were identified as BYI 02960-OH and BYI 02960-difluoroethyl-amino-furanone. The identification rates amounted to approx. 96% of the total radioactivity in males and 100% in females. The detailed results of the metabolic profiles of the plasma samples from all tests are summarized in Table 5.1.2-16.

Table 5.1.2.16: Concentration of parent compound and metabolites in plasma of male and female rats sacrificed 6 h after single oral administration of 3 mg/kg [furanone-4-^{14}C]BYI 02960.
Data are presented as mg/kg

	Male	Female
Sampling period [h]	**0 - 6 h**	**0 - 6 h**
Parent compound	1.087	1.385
unknown	0.056	0.007
unknown	-	0.010
BYI 02960-difluoroethyl-amino-furanone	0.101	0.012
BYI 02960-des-difluoroethyl	-	0.008
BYI 02960-OH	0.068	0.026
Total identified	1.257	1.369
Total characterized *	0.056	0.016
Sum total	**1.313**	**1.385**

* : Peaks were characterized based on their retention time in HPLC-analysis

3. Metabolites in liver

In the liver extract of male and female rats, the parent compound was the dominating component (males: 2.11 mg/kg, females: 2.78 mg/kg). The metabolites identified were BYI 02960-difluoroethyl-amino-furanone, BYI 02960-OH-gluA, BYI 02960-des-difluoroethyl and BYI 02960-OH. Their concentrations ranged from 0.015 mg/kg to 0.177 mg/kg. Additionally, another five minor metabolites were found which were not identified. The identification rates of parent compound and metabolites ranged from approx. 88% of the total radioactivity in males to approx. 99% in females. The detailed results of the metabolic profiles in the liver extracts of male and female rats are summarized in Table 5.1.2-17.

Table 5.1.2-17: Concentration of parent compound and metabolites in the liver of male and female rats sacrificed 6 h after single oral administration of 3 mg/kg [furanone-4-^{14}C]BYI 02960.
Data are presented as mg/kg

	Male	Female
Sampling [h]	**6 h**	**6 h**
Parent compound	2.110	2.778
unknown	0.150	---
unknown	0.015	---
BYI 02960-difluoroethyl-amino-furanone	0.108	0.015
BYI 02960-OH-gluA (isomer 1)	0.026	---
BYI 02960-OH-gluA (isomer 3)	0.055	0.013
BYI 02960-des-difluoroethyl	0.036	0.024
unknown	0.012	---
unknown	0.007	---
unknown	0.016	---
BYI 02960-OH	0.242	0.077
Total identified	2.577	2.908
Total characterized *	0.200	---
Sum total	**2.928**	**2.937**

* : Peaks were characterized based on their retention time in HPLC-analysis

4. Metabolites in kidney

In the kidney extract of male rats, the parent compound was the dominating component and accounted for 1.969 mg/kg. BYI 02960-difluoroethyl-amino-furanone and the glucuronic acid conjugates of BYI 02960-OH were identified as metabolites. Their concentrations ranged from 0.010 to 0.319 mg/kg. Additionally, another two unknown minor metabolites were found. The parent compound was also the main constituent of the kidney extract of female rats at a concentration of approx. 4.03 mg/kg. The identified metabolites were BYI 02960-des-difluoroethyl and BYI 02960-OH. Their concentrations ranged from 0.047 mg/kg to 0.197 mg/kg. The identification rates ranged from approx. 91% in males to 98% of the total radioactivity in females. The detailed results of the metabolic profiles in the kidney extracts are summarized in Table5.1.2-18.

Table 5.1.2-18: Concentration of parent compound and metabolites in the kidney of male and female rats sacrificed 6 h after single oral administration of 3 mg/kg [furanone-4-^{14}C]BYI 02960.
Data are presented as mg/kg

	Male	Female
Sampling [h]	**6 h**	**6 h**
Parent compound	1.969	4.025
unknown	0.174	0.060
unknown	0.017	
BYI 02960-difluoroethyl-amino-furanone	0.125	---
BYI 02960-OH-gluA (isomer 1)	0.010	---
BYI 02960-OH-gluA (isomer 3)	0.020	---
BYI 02960-des-difluoroethyl	0.036	0.047
BYI 02960-OH	0.319	0.197
Total identified	2.479	4.269
Total characterized *	0.191	0.060
Sum total	**2.732**	**4.346**

* : Peaks were characterized based on their retention time in HPLC-analysis

5. Metabolites in muscle

In the muscle extract of male rats, the parent compound was the dominating component and accounted for 1.16 mg/kg. BYI 02960-difluoroethyl-amino-furanone, BYI 02960-des-difluoroethyl and BYI 02960-OH were identified as metabolites. Their concentrations ranged from 0.013 to 0.090 mg/kg. Additionally, another unknown minor metabolite was found. The parent compound was also the main constituent of the muscle extract of female rats at a concentration of approx. 1.44 mg/kg. The identified metabolites were BYI 02960-difluoroethyl-amino-furanone, BYI 02960-des-difluoroethyl and BYI 02960-OH. Their concentrations ranged from 0.009 mg/kg to 0.032 mg/kg. The identification rates ranged from approx. 97% in males to approx. 100% of the total radioactivity in females. The detailed results of the metabolic profiles in the kidney extracts are summarized in Table5.1.2-19.

Table 5.1.2-19: Concentration of parent compound and metabolites in muscle of male and female rats sacrificed 6 h after single oral administration of 3 mg/kg [furanone-4-^{14}C]BYI 02960.
Data are presented as mg/kg

	Male	Female
Sampling [h]	**6 h**	**6 h**
Parent compound	1.160	1.435
unknown	0.026	---
BYI 02960-difluoroethyl-amino-furanone	0.090	0.009
BYI 02960-des-difluoroethyl	0.013	0.012
BYI 02960-OH	0.083	0.032
Total identified	1.346	1.487
Total characterized *	0.026	---
Sum total	**1.382**	**1.489**

* : Peaks were characterized based on their retention time in HPLC-analysis

6. Metabolites in fat

In the fat extract of male rats, the parent compound was the dominating component and accounted for 0.474 mg/kg. BYI 02960-difluoroethyl-amino-furanone (0.032 mg/kg) and BYI 02960-OH (0.040 mg/kg) were identified as metabolites. The parent compound was the only component identified in the fat extract of female rats (approx. 0.65 mg/kg). The identification rates of parent compound and metabolites ranged from approx. 96% in males to approx. 100% of the total radioactivity in females.
The detailed results of the metabolic profiles in the fat extracts from all tests are summarized in Table 5.1.2-20.

Table 5.1.2-20: Concentration of parent compound and metabolites in the fat of male and female rats sacrificed 6 h after single oral administration of 3 mg/kg [furanone-4-^{14}C]BYI 02960.
Data are presented as mg/kg

	Male	Female
Sampling [h]	**6 h**	**6 h**
Parent compound	0.474	0.650
unknown	---	---
BYI 02960-difluoroethyl-amino-furanone	0.023	---
BYI 02960-OH	0.040	---
Total identified	0.537	0.650
Sum total	**0.558**	**0.651**

E. Comparison of the Metabolic Profiles

Six hours after administration BYI 02960 was only incompletely metabolised. Metabolic reactions took place at least at 3 different structural positions of the molecule. The majority of the radioctive residues were identified (approx. 89 - 100% in plasma and in extracts of organs and tissues as well as ≥ 88% of radioactivity in urine samples).
In all samples of plasma, organs and tissues the parent compound was the by far largest component accounting for more than 72% of the total radioactivity. None of the identified metabolites accounted for more than 12% of the total radioactivity. Also in urine, the parent compound was the dominating radioactive component (approx. 22% of the dose in males and 38% in females).
The metabolism was qualitatively similar in male and female rats, but with quantitative differences. The degradation of the parent compound to the different metabolites was significantly higher in male as compared to female rats.

F. Biotransformation Pathway

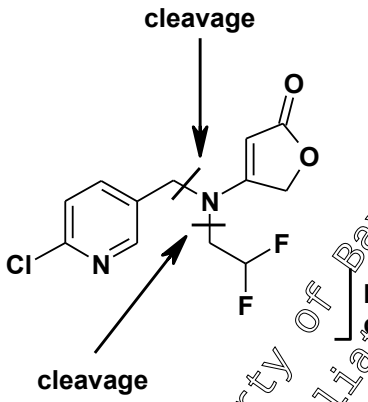
The principal metabolic reactions of [furanone-4-^{14}C]BYI 02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid,
- cleavage of the difluoroethyl group leading to BYI 02960-des-difluoroethyl and,
- cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

Figure 5.1.2.07 schematically shows the positions in the molecule, which are involved in these metabolic reactions.

The proposed biotransformation pathway of [furanone-4-^{14}C] BYI 02960 is presented in Figure 5.1.2-08.

Figure 5.1.2-07: Positions involved in metabolic degradation of BYI 02960



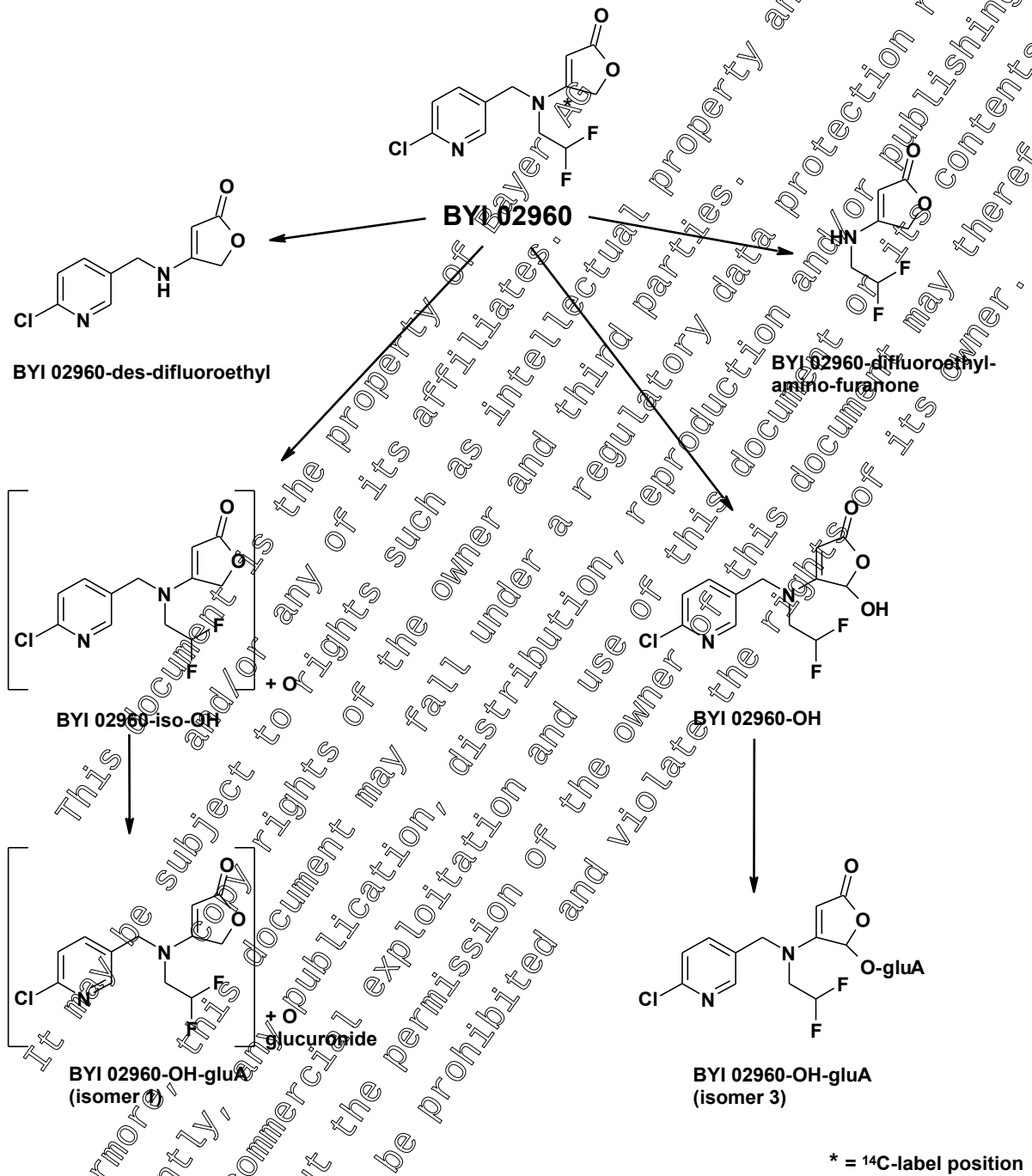
G. Conclusions

The kinetic and metabolic behavior of [furanone-4-^{14}C]BYI 02960 in male and female Wistar rats can be characterized by the following observations:

- The distribution of the radioactivity within the organs and tissues (*i.e.* blood, liver, kidney, muscle and fat) showed a distinctive preference for liver and kidney as the main metabolizing and excretory organs.
- Parent compound, major and several minor metabolites were identified in all samples. Identification rates were high with ≥ 88% of radioactivity in urine and approx. 89 - 100% of the radioactivity in plasma, organs and tissues.
- The metabolic transformation of BYI 02960 was principally oxidative in nature and took place at least at 3 different structural positions of the molecule.
- The metabolic pattern was in good accordance with that obtained from the corresponding ADME rat study (KIIA 5.1.2/01). With regard to the extent of metabolism, a clear sex difference was observed since it was higher in male than in female rats, *i.e.* the metabolic degradation of the parent compound was much less pronounced in females.

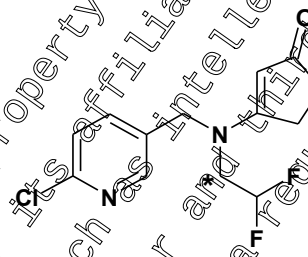
Many examples of sex differences of metabolism in rats have been reported in the literature. A very important and general observation is the approximately threefold difference in the activity of hepatic microsomal monooxygenase (cytochrome P-450) in male as compared to female rats. These results are possibly due to the effects of hormones (sex, growth, and thyroid hormones) but also by other chemicals and have been known for a long time (references are provided in the report).

Figure 5.1.2-08: Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in male and female rats based on metabolites detected in urine, plasma, liver, kidney, muscle and fat



KIIA 5.1.3 - Toxicokintic studies - Repeated dose, oral route, in rats

Two studies are summarised in this chapter. The first study report (No. MEF-11/555) describes the absorption, distribution, metabolism, and excretion of the ethyl-1-^{14}C labelled test compound in male rats which were dosed with a single oral low dose. The excretion of radioactivity was investigated in urine, faeces and expired air, the radioactivity concentration was determined in the organs and tissues at sacrifice and the metabolites were identified in the excreta. The toxicokinetic behaviour of the total radioactivity was investigated by plasma curve analysis.
The second study (Report No. MEF-11/270) describes the distribution and excretion of the ethyl-1-^{14}C labelled test compound in male and female rats after a single oral low dose. The distribution and metabolism of the administered radioactivity was determined in urine as well as in plasma, liver, kidney, muscle and fat at sacrifice at three different time points.
The position of the radiolabel is shown in this figure:

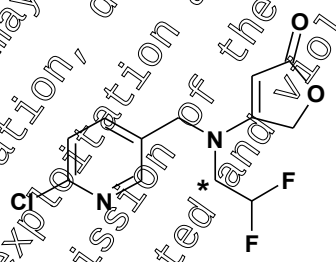


[ethyl-1-^{14}C]BYI 02960

Report:	**KIIA 5.1.3/01, [redacted]; 2011**
	[Ethyl-1-^{14}C]BYI 02960 – Absorption, Distribution, Excretion, and Metabolism in Male Rats
Report No & Document No	MEF 11/555 M-415647-01-1
Guidelines:	OECD Guideline for Testing Chemicals, 417: Toxicokinetics US EPA Health Effects Test Guidelines OPPTS 870.7485, Metabolism and Pharmacokinetics PMRA Regulatory Directive Dir2005-01: Section 11: Toxicokinetic studies Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	Yes, fully compliant US EPA – FIFRA Good Laboratory Practice (40 CFR Part 160) Principles of Good Laboratory Practice – German Chemical Law (Chemikaliengesetz), dated 2002-06-20, current version of Annex 1 JAPAN MAFF - Notification on the Good Laboratory Practice Standards for Agricultural Chemicals (11 Nousan 6283) notified 1999-10-01
Testing Facility and Dates	[redacted] Experimental work: 2010-01-21 - 2010-11-12

Executive Summary

The absorption, distribution, excretion, and metabolism of the new insecticide BYI 02960 (common name: flupyradifurone) were investigated in male Wistar rats. Four animals were orally administered with a single dose of BYI 02960 at a dose level of 2 mg/kg bw. The test compound was radiolabelled with ^{14}C in the 1-position of the ethyl side chain:



*** denotes the position of the ^{14}C-label**

The animals were sacrificed 72 h after dosing. Expired air, urine, and faeces were collected between dosing and sacrifice. The radioactivity level in plasma was followed by collection of micro samples from each animal at 12 time points. The total radioactivity was determined in excreta and in organs and tissues collected at sacrifice. Metabolism was investigated in urine and faeces samples.
The total recovery was almost quantitative since approx. 100% of the administered dose was found in the excreta and in the body at sacrifice. [Ethyl-1-^{14}C]BYI 02960 was almost completely absorbed because ≥85% of the administered dose was detected in the urine and the body without GIT at sacrifice. The absorption commenced immediately after dosing as can be seen from the fast increase of radioactivity in plasma samples within the first hour.

The distribution of the radioactivity within the body was fast and the maximum plasma concentration (C_{max}) was reached within one hour after administration. From the maximum, the radioactivity level declined slowly to ca. 50% of C_{max} after 8 hours and ca. 8% of C_{max} at the time of sacrifice.
Excretion of radioactivity was fast and mainly renal. Approximately 82% of the dose was excreted with the urine and ca. 14% with the faeces. Only a negligible part of 0.2% of the dose was detected in expired air. The major part of the dose (>87%) was excreted within 24 hours after treatment. Excretion was continuing until sacrifice. In particular, the major part of the metabolite BYI 02960-DFA was excreted on day 2 and day 3.
At the time of sacrifice ca. 3% of the dose was detected in the body without GIT. The residue concentration was highest in plasma with 0.158 mg/kg. For most other organs and tissues, levels were in the range between 0.05 and 0.1 mg/kg. Metabolic profiles in urine and extracts of faeces were determined by reversed phase HPLC with radiodetection using a neutral water/acetonitrile gradient. Parent compound, one major and five minor metabolites were identified by HPLC and/or TLC co-chromatography. The label-specific metabolite BYI 02960-DFA was additionally identified by high resolution LC/MS of the isolated compound. Identification rates were >95% of the total radioactivity in urine and >85% of the total radioactivity in faeces.
Approx. 92% of the total dose administered was identified in the excreta. Another ca. 3% of the dose corresponding to 67 unknown metabolites was characterized by their chromatographic behavior. All metabolites representing >1% of the dose administered have been identified.
Metabolism results as percent of the total dose administered are summarized in the Table 5.1.3-01:

Table 5.1.3-01: Amounts of identified metabolites expressed as % of the total dose in the excreta of male rats after oral administration of 2 mg/kg [ethyl-1-^{14}C]BYI 02960

(BYI 02960-)	Urine	Faeces	Total
DFA	5.28	0.49	5.77
difluoroethyl-amino-furanone	3.63	---	3.63
OH-gluA (isomer 1)	1.40	---	1.40
OH-gluA (isomer 3)	1.79	---	1.79
OH	16.13	7.60	23.73
parent	51.96	3.79	55.75
iso-OH	---	0.43	0.43
Identified	80.19	12.31	92.50
Characterized	2.04	0.27	2.31
Total	**82.23**	**13.51**	**95.74**

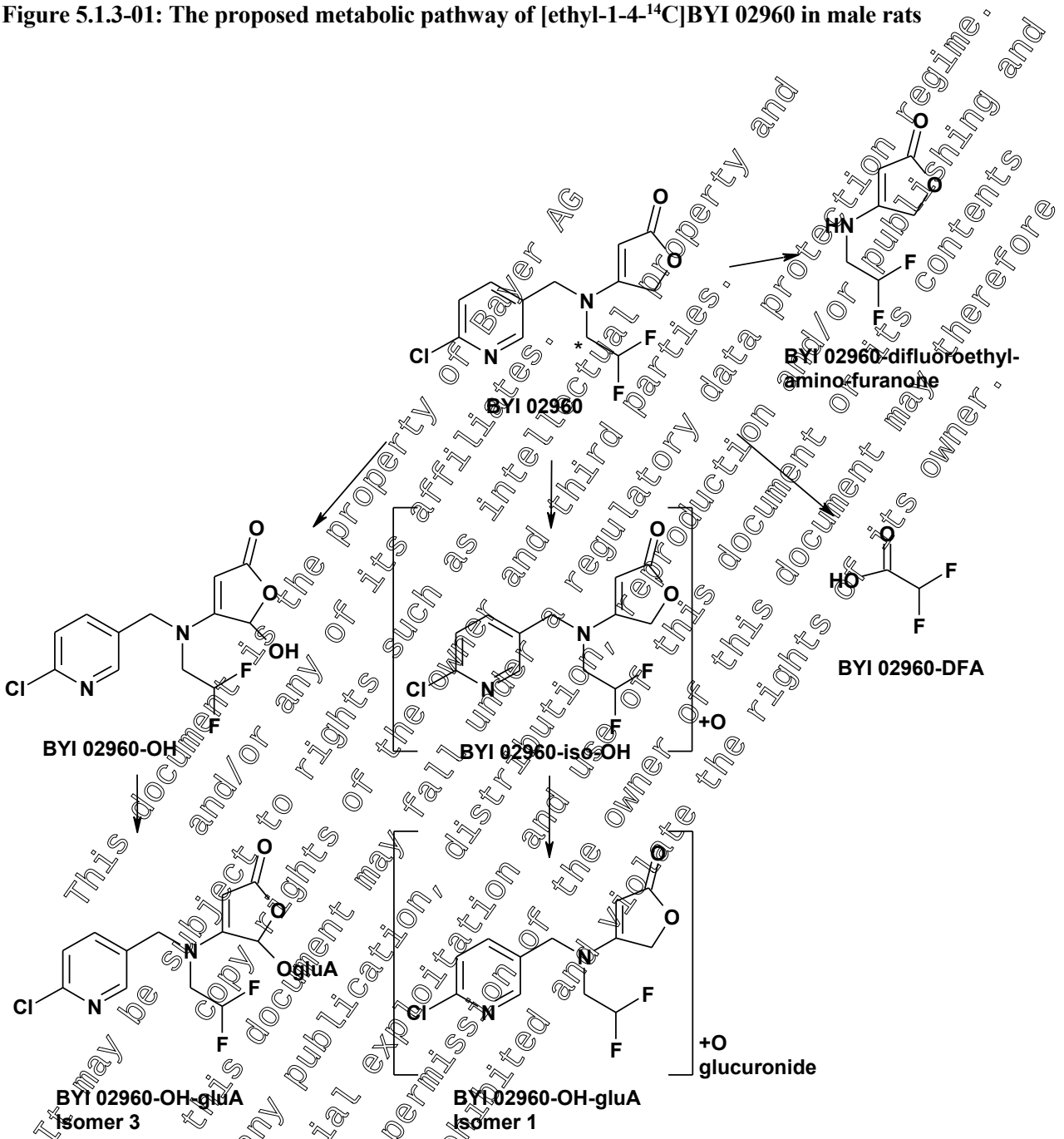
The principal metabolic reactions of [ethyl-1-^{14}C]BYI 02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid,
- cleavage of the difluoroethyl group leading to BYI 02960-DFA and
- cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

The results of the metabolism investigations are in good accordance with those obtained in the corresponding organ metabolism rat study (KIIA 5.1.3/02).

The proposed metabolic pathway of [ethyl-1-^{14}C]BYI 02960 in rats is shown in Figure 5.1.3-01:

Figure 5.1.3-01: The proposed metabolic pathway of [ethyl-1-4-^{14}C]BYI 02960 in male rats



I. Material and Methods

A. Material

1. Test Material:

IUPAC Name:	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name:	BYI 02960
Common name:	Flupyradifurone
Empirical formula:	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass:	288.68 g/mol
Water solubility:	pH 4 = 3.2 mg/L, pH 7 = 3.2 mg/L pH 9 = 3.0 mg/L
n-Octanol/water partition coefficient:	pH 4 = 1.2, pH 7 = 1.2, pH 9 = 1.2
Labelling:	[ethyl-1-^{14}C]
Specific radioactivity of the radiolabelled batches:	3.93 MBq/mg = 2.36 x 10^5 dpm/µg = 106.28 µCi/mg = 30.68 Ci/mol
Specific radioactivity used for administration:	3.93 MBq/mg = 2.36 x 10^5 dpm/µg = 106.28 µCi/mg = 30.68 Ci/mol
Radiochemical purity:	> 99 % (certified, HPLC and TLC with radiodetection)
Dose level:	2 mg/kg body weight
Vehicle:	0.5 % aqueous tragacanth suspension
Stability of the test material:	The stability of [ethyl-1-^{14}C]BYI 02960 was determined by radio-HPLC of the administration suspensions immediately after dosing

2. Test Animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	Wistar Hsd/Cpb: WU
Breeding facility:	[redacted]
Sex and numbers involved:	4 male animals
Age:	6 weeks at the time of delivery
Body weight:	197 - 201 g at the time of administration
Acclimatization:	Makrolon® cages on wood shavings in the test facility for 7 days prior to the administration
Identification:	Cage cards on which the study number, test compound name and individual animal number were displayed. Additional labelling with water-insoluble spots on the tail
Housing:	After administration of the radiolabelled test item individually in Makrolon® metabolism cages under conventional hygienic conditions in air-conditioned rooms Temperature 22 - 24 °C, relative humidity 43 - 51 %. 12 / 12 hours light / dark cycle, air change 10 - 15 times per hour

Feed and water:	Rat/mice maintenance long life diet (no. 3883.0.15), supplied by Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland (ca. 16 g per animal and day) Last feeding ca. 16 h prior to dosing Next feeding ca. 6 h after dosing Tap water from municipal water supply, *ad libitum*

B. Study Design

1. Dosing

Each rat orally received 2 mg/kg bw of [ethyl-1-^{14}C]BYI 02960 suspended in 0.5 % aqueous tragacanth after ca. 16 h of fasting. The dosing suspension (0.15 mg/mL) was prepared one day before dosing and stored at 5°C.

The suspension was administered by oral gavage using a syringe attached to an animal-feeding knob cannula. Each animal received a volume of 2 mL (10 mL/kg bw calculated for a nominal body weight of 200 g). The individual amounts of the administered test substance were calculated by dividing the amount of the administered radioactivity by the specific radioactivity. The actual mean administered dose of [ethyl-1-^{14}C]BYI 02960 was 2.09 mg/kg bw. The stability of the test compound in the suspension was assured by radio-HPLC analysis after administration.

2. Collection of excreta

After administration the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine, faeces, and expired air. Urine was collected separately for each animal in a cryogenic trap cooled with dry ice in intervals of 4 h, 8 h, 12 h, 24 h, 48 h and 72 h. The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction. The radioactivity was determined by LSC.

The faeces samples were collected every 24 h separately for each animal before they were lyophilised, weighed, and homogenised. The radioactivity was determined by combustion/LSC.

3. Trapping of expired air

Carbon dioxide and other volatiles from expired air were collected in intervals of 0 - 24 h, 24 - 48 h and 48h - 72h. The metabolism cages were connected to a high velocity air pump and ventilated with ca. 3 L of air per minute and cage. The air was passed through a trapping system of two gas-washing bottles. The first one containing ca. 250 mL of aqueous ammonia (10%) and the second containing about 250 mL of a 1:1-mixture (v/v) of ethanolamine/ethanol. At sampling, the exact volume was determined, from which an aliquot was taken for the determination of radioactivity by LSC.

4. Plasma micro-samples

Blood samples were collected separately for each animal by pressing a capillary coated with heparin in a small cut in the tail vein. The wound was sealed with adhesive tape. The capillaries were centrifuged using a hematocrit centrifuge to separate plasma from the formed blood constituents. After centrifugation, the capillary was broken at the border between plasma and formed constituents and the plasma (approx. 10 - 100 mg) squeezed out onto a small metal dish for weighing. After weighing, this dish was placed into a scintillation vial for radioactivity measurement. Blood samples were collected at

10 min, 20min, 40 min, 1 h, 1.5 h, 2 h, 4 h, 8 h, 24 h, 32 h, 48 h, and 72 h after dosing from the same animals. Thus single animal plasma curves were generated avoiding inter-animal variations. For pharmacokinetic calculations, the average plasma value of the four rats was used.

5. Sacrifice

The rats were sacrificed in Pentobarbital-Na anaesthesia (Narcoren®, supplied by [redacted] Germany) by transection of the cervical vessels and exsanguinated.

6. Plasma, tissues and organs at sacrifice

At sacrifice, blood was collected in heparinised test tubes and separated into plasma and blood cells by centrifugation. After weighing, aliquots were taken for determination of radioactivity by LSC. Organs and tissues were weighed immediately after dissection and again after lyophilisation. Finally, they were homogenized prior to taking aliquots for the determination of radioactivity by combustion/LSC. For small organs and tissues such as adrenal glands, thyroid and renal fat, only the wet weight was determined before they were solubilised using BTS 450 (Beckman Tissue Solubiliser) and radioassayed by LSC. The radioactivity remaining in the GIT, skin, and residual carcass was determined by combustion/LSC in order to establish the radioactivity balance. The gastro-intestinal tract and an aliquot of depilated skin were lyophilised. After weighing and homogenization, aliquots were taken for determination of radioactivity by combustion/LSC.

7. Measurement of radioactivity

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). All solid samples were combusted in an oxygen atmosphere using an oxidizer. The released $^{14}CO_2$ was trapped an an alkaline scintillation cocktail and the radioactivity was determined by LSC.

8. Toxicokinetic analysis

In this study the software TOPFIT (ver. 2.0) was used to calculate the toxicokinetic parameters by plasma concentration-time curve analysis for the mean equivalent concentration values. A standard 2-compartment disposition model was applied for curve fitting. Compartment models attempt to mathematically describe the processes of absorption, entry into the systemic circulation, distribution to organs or tissues where metabolism can occur, and subsequent excretion.

9. Analytical methods

Samples were analyzed by radiochromatographic (HPLC, TLC) and spectroscopic (LC-MS, ^{1}H-NMR) methods.

10. High Performance Liquid Chromatography (HPLC)

HPLC-radioactivity analysis was performed on an Agilent 1100 system with radiometric- and UV-detection. The separation was carried out on a reversed phase column using a neutral water / acetonitrile gradient. Detailed information of the HPLC-methods and instruments set-up can be found in the report. All solvents were of HPLC-quality.

The chromatograms were recorded and quantitatively evaluated using the software package GINA® (raytest, Benzstraße 4, D-75334 Straubenhardt, Germany). The ^{14}C-trace of a chromatogram was divided into regions of interest (ROI's), according to the separated radioactive peaks. Additionally,

background regions (BKG's) were defined and used for background correction. The background corrected area counts from all regions of interest were used for calculation of the quantitative distribution of the individual components in the sample. A quantifiable radioactive peak was regarded relevant if resulting in a signal approx. 2.5 times above the background noise.
For co-chromatography in HPLC, the sample was mixed with the reference compound before injection. The detection was achieved either by UV-absorption of the non-radiolabelled or by ^{14}C-detection of the radiolabelled reference compound. Chromatographic matching with the non-radiolabelled reference compound was assessed by comparison of the UV-trace and the associated ^{14}C-trace. Chromatographic matching with the radiolabelled reference compound was assessed by comparison of the ^{14}C-chromatogram of the mixture with the ^{14}C-chromatogram of the sample without the reference compound.

11. Thin Layer Chromatography (TLC)
For detection and identification of parent compound and BYI 02960-DFA in urine and faeces extracts, thin-layer chromatography (silica 60 F_{254}, normal phase) was used. The samples were spotted on the TLC-plates using a Linomat IV (Camag, Berlin, Germany). The plates were developed using ethyl-acetate / isopropanol / water / acetic acid (65/24/11/1, v/v/v/v) as a solvent system.
The chromatograms were visualized under UV light (254 nm). The radioactive zones were detected using a Fujibas® 2000 bio-imaging system (Fuji, Japan & raytest, Straubenhard, Germany). Fujibas® imaging plates were exposed to the TLC-plates. The imaging data were identified with Basreader software (version 2.13e, raytest, Straubenhard, Germany). Evaluation and visualization of recorded data was performed using the AIDA software (raytest, Straubenhard, Germany). The detection limit (LOD) was 5 - 10 dpm/zone for ^{14}C after an exposure of at least 14 hours.
For co-chromatography in TLC, a solution of the reference compound was applied to the plate as a 1 to 1.5 cm-wide band. The sample solution was also applied as a 1 to 1.5 cm-wide band, part of which (ca. 5 to 8 mm) overlapped with the reference compound band. After development, chromatographic matching with the reference compound was assessed by analysis of the individual radioluminogram.

12. Mass spectroscopy
Electro-spray ionization MS spectra (ESI) were obtained either with a TSQ 7000 instrument or with a LTQ Orbitrap XL mass spectrometer (Thermo or Finnigan, respectively, San Jose, CA, U.S.A.). The HPLC instrument used for chromatography was an Agilent HP1100 (Agilent, Waldbronn, Germany). The eluate of the HPLC-column was split between the UV-detector followed by a radioactivity detector (Ramona Star, Raytest, Straubenhardt, Germany) and the MS spectrometer.

13. ^{1}H-NMR spectroscopy
600 MHz ^{1}H-NMR spectra were recorded on a BRUKER AV 600 instrument (Bruker, Karlsruhe, Germany).

14. Preparation of samples, extraction and analysis
One half of the urine and faeces samples from all four animals were combined to a pool sample for each test group and time period. The amount of radioactivity applied for analysis was calculated from the in-life data of the respective samples. Aliquots of the urine samples were taken without further sample processing for metabolic profiling by HPLC. The combined faeces samples were extracted with acetonitrile/water mixtures. The extracts containing the major part of the radioactive residue were

combined, further purified by SPE on a C18 cartridge and concentrated for subsequent HPLC analysis. The radioactivity in the post extraction solids was determined by combustion analysis followed by LSC.

15. Identification / Characterization and quantification of residues

The identification of metabolites was mainly achieved by comparison of the chromatographic profile of the urine samples with results of the organ metabolism study with [ethyl-1-^{14}C]BYI 02960 (KIIA 5.1.3/02). The label-specific metabolite BYI 02960-DFA was isolated from urine by repeated HPLC fractionation and subsequent subjection to high resolution LC/MS analysis. Additional identification of parent compound and the DFA-metabolite was performed by HPLC and TLC co-chromatography. Based on the identification, all chromatograms were integrated and corresponding peaks in all samples were assigned the same peak number.

II. Results and Discussion

A. Recovery

The total recovery was almost quantitative since approx. 100% of the administered dose was found in the expired air, the excreta, and the body at sacrifice. The results in percent of the given dose in expired air, urine, faeces, organs and tissues at sacrifice are summarized in Table 5.1.3-02 (below).

Table 5.1.3.02: Recovery of radioactivity in urine, faeces, gastrointestinal tract and the body following a single oral dose of 2 mg/kg [ethyl-1-^{14}C]BYI 02960

	% of given dose
Expired air	0.20
Faeces	13.63
Urine	82.24
Sum of excreta	**95.95**
Body excluding GIT	3.19
GIT	0.73
Total Body	3.92
Balance	**99.86**

B. Absorption

[Ethyl-1-^{14}C]BYI 02960 was almost completely absorbed in male rats. The absorption rate was at least 85% because >82% of the dose was detected in urine and >3% in the body without GIT. The absorption commenced immediately after dosing as can be seen from the quick increase of radioactivity in plasma micro samples (see Table 5.1.3-03 below).

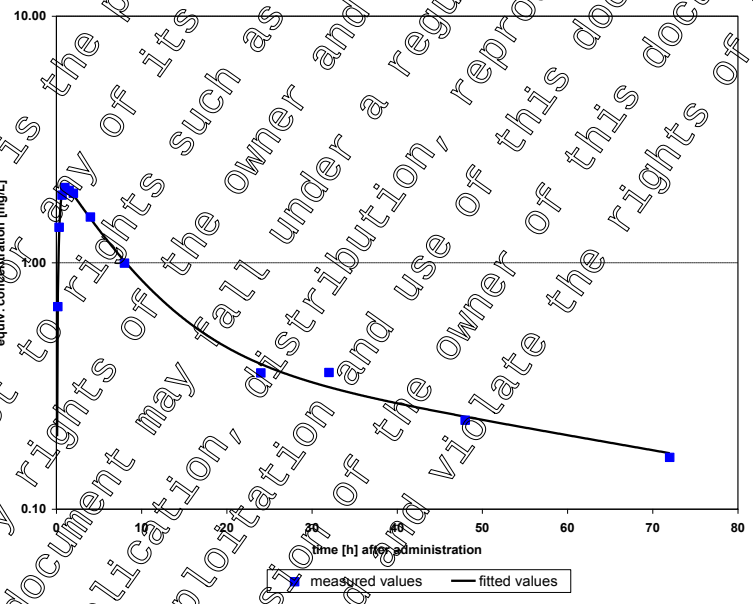
Table 5.1.3.03: Timecourse of radioactivity in the plasma following a single oral dose of 2 mg/kg [ethyl-1-^{14}C] BYI 02960

Time point	Equivalent concentration (mg/kg)
10 min	0.663
20 min	1.391
40 min	1.878
1 h	2.017
1 h, 30 min	1.963
2 h	1.911
4 h	1.532
8 h	0.995
24 h	0.357
32 h	0.358
48 h	0.230
72 h	0.162

C. Distribution and plasma kinetics

The test compound was quickly distributed as can be seen form the analysis of plasma at different time points. The maximum concentration of radioactivity (C_{max}) was already reached one hour after administration. At this time, the radioactivity level in plasma corresponded approximately to the equidistribution concentration.
The plasma concentration then declined to approx. 50% of C_{max} within 8 hours and to approx. 10% of the maximum value within 48 hours. At the time of sacrifice, the plasma concentration amounted to ca. 8% of C_{max}. (see Table 5.1.3-03).
The mean values of total radioactivity were used for a toxicokinetic modelling using the TOPFIT software. The fitting was conducted assuming a two-compartment model with no data weighting. A good fit was achieved for the entire time range. The AUC was 45.4 mg/L*h and the half life of elimination was 49.9 hours.
The corresponding concentration-time curve of the modelled data and the measured data is shown in Figure 5.1.3-02 below.

Figure 5.1.3-02: Timecourse of radioactivity in the plasma following a single oral dose of 2 mg/kg [ethyl-1-^{14}C] BYI 02960



D. Excretion

Only a very small proportion of the dose (0.2% in total) was detected in the expired air. This result confirms the stability of the radio label in the ethyl group with regard to extensive metabolic transformation. The major route of excretion was renal. In total, approx. 82% of the dose was detected in urine, the majority of which (ca. 76%) was excreted within 24 hours. Faecal excretion accounted for ca. 13.5% of the given dose. Also the major part of faecal residues (ca. 11%) was excreted within the first day after treatment. These results are in good accordance with the excretion behavior detected in all other rat studies. The cumulative excretion results in percent of the administered radioactivity are summarized in Table 5.1.3-04.

Table 5.1.3-04: Cumulative excretion of radioactive residues via urine, faeces and expired air after a single oral dose of 2 mg/kg [ethyl-1-^{14}C]BYI 02960

	Time [h]	% of dose
Expired Air	24	0.15
	48	0.19
	72	0.20
Faeces	24	11.08
	48	13.12
	72	13.51
Urine	4	14.95
	8	46.40
	12	60.48
	24	76.12
	48	80.60
	72	82.24
Total		**95.95**

E. Radioactive residues in organs and tissues at sacrifice

Approx. 4% of the dose was detected in the body at sacrifice 72 hours after oral administration; 0.73% was found in the GIT and 3.19% in the body without GIT. Residual concentration of radioactivity was in the range of 0.025 to 0.158 mg/kg. The lowest concentration was detected in the Harderian gland and the highest value was found in plasma. However, concentrations for most organs and tissues were very similar and in the range of approx 0.05 to 0.1 mg/kg. The equivalent concentrations of the residual radioactivity in organs and tissues at sacrifice are presented in Table 5.1.3-05.

Table 5.1.3-05: Total radioactive residues in organs and tissues at sacrifice after a single oral dose of 2 mg/kg [ethyl-1-^{14}C]BYI 02960

	Equivalent concentration (mg/kg)
Blood cells	0.104
Plasma	0.158
Carcass	0.064
Heart	0.078
Brain	0.083
Kidneys	0.066
Liver	0.095
GIT	0.129
Testes	0.065
Adrenal gland	0.073
Harderian gland	0.025
Thyroid	0.088
Spleen	0.075
Lung	0.088
Eye	0.138
Skin	0.079
Bone (femur)	0.052
Perirenal fat	0.054
Muscle	0.055

F. Identification / characterisation and quantification of residues

The following strategy was used for the identification of parent compound and metabolites:
The chromatographic profile of the urine sample 12 - 24h was compared with the profile of the urine pool 0 - 24h of the organ metabolism study using [ethyl-1-^{14}C]BYI 02960 (KIIA 5.1.3/02) for a first assignment of parent compound and metabolites. In this study all major compounds in urine were identified by LC-MS/MS and/or HPLC and TLC co-chromatography.
The metabolite BYI 02960-DFA was isolated from the urine pool 24 - 48h and identified by high resolution LC-MS. Parent compound, BYI 02960-DFA, and BYI 02960-iso-OH were additionally identified by HPLC and TLC comparison and/or co-chromatography with selected samples.

G. Metabolites in urine and faeces

1. Metabolism in urine

The main compound in urine was unchanged BYI 02960 representing more than 50% of the given dose. Only one major metabolite was detected, BYI 02960-OH, which accounted for ca. 16% of the dose. BYI 02960-DFA was found at a level of ca. 5% of the dose. This metabolite was predominantly excreted between 24 and 72 hours after administration. Other identified metabolites were BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH-gluA (isomer 1 and 3), each accounting for less than 4% of the dose.

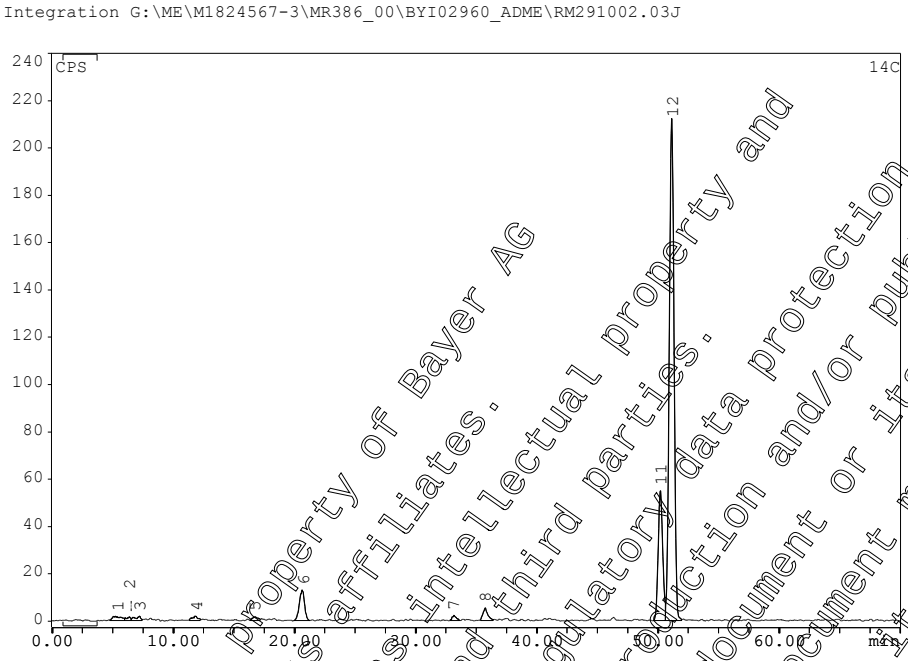
In total, ca. 82% of the given dose was detected in urine of which ca. 80% was identified and ca. 2% characterized. Each of the four characterized metabolites made up for less than 1% of the administered dose. The results are summarized in Table 5.1.3-06. The peak numbers in the table correlate with those in the metabolic profile shown in Figure 5.1.3-03.

Table 5.1.3-06: Amounts of metabolites in the urine after a single oral dose of 2 mg/kg [ethyl-1-^{14}C]BYI 02960. The data are given in % of the dose

Peak no.	(BYI 02960-)	0-4 h	4-8 h	8-12 h	12-24 h	24-48 h	48-72 h	Urine Total
1	DFA	---	0.36	0.43	1.06	1.78	1.64	5.28
2	unknown 1	---	0.14	0.30	0.20	---	---	0.64
3	unknown 2	---	0.24	0.23	0.22	---	---	0.68
4	unknown 3	---	0.25	0.11	---	---	---	0.36
5	unknown 4	---	0.21	0.16	---	---	---	0.37
6	difluoroethyl-amino-furanone	0.42	1.50	0.91	0.66	0.14	---	3.63
7	OH-gluA (isomer 1)	0.17	0.20	0.29	0.50	0.25	---	1.40
8	OH-gluA (isomer 3)	0.33	0.57	0.30	0.44	0.15	---	1.79
11	OH	2.49	5.51	3.31	3.77	1.05	---	16.13
12	parent	11.54	22.46	8.05	8.79	1.11	---	51.96
	Total	**14.95**	**31.45**	**14.08**	**15.64**	**4.48**	**1.64**	**82.23**
	Identified	**14.95**	**30.61**	**13.29**	**15.22**	**4.48**	**1.64**	**80.19**
	Characterized	**---**	**0.84**	**0.79**	**0.42**	**---**	**---**	**2.04**

A representative chromatogram of the sampling interval 4 - 8 h is shown in Figure 5.1.3-03.

Figure 5.1.3-03: Metabolic profile of urine 4 - 8 h of male rats after a single oral dose of 2 mg/kg [ethyl-1-^{14}C]BYI 02960



2. Metabolism in faeces

The unchanged parent compound represented ca. 4% of the given dose in faeces. The main constituent of the faecal radioactivity was BYI 02960-OH accounting for ca. 8% of the dose. BYI 02960-DFA and BYI 02960-iso-OH were found at levels of ca. 0.5% of the dose. In total, ca. 13.5% of the dose was detected in faeces, ca. 12% thereof was identified and ca. 0.3% characterized. The two characterized metabolites were found at levels <0.2% of the given dose. The results are summarized in Table 5.1.3-07.

Table 5.1.3.07: Amounts of metabolites in the faeces after a single oral dose of 2 mg/kg [ethyl-1-^{14}C]BYI 02960. The data are given in % of the dose

Peak no.		0-24h	24-48 h	48-72 h	Faeces Total
1	BYI 02960-DFA	0.20	0.29	---	0.49
9	unknown 5	0.10	---	---	0.10
10	unknown 6	0.17	---	---	0.17
11	BYI 02960-OH	6.26	1.34	---	7.60
12	Parent compound	3.64	0.15	---	3.79
13	BYI 02960-iso-OH	0.27	0.16	---	0.43
	Total in extracts	10.64	1.94	---	12.58
	Identified	10.38	1.94	---	12.31
	Characterized	0.27	---	---	0.27
Total in faeces		**11.08**	**2.04**	**0.39**	**13.51**

H. Comparison of the metabolic profiles in urine and faeces

BYI 02960 was metabolized to a number of metabolites. The unchanged parent compound represented the predominant part of the radioactivity in urine while in faeces the metabolite BYI 02960-OH was more prominent. BYI 02960-DFA was detected in both, urine and faeces. All other identified and characterized metabolites represented a minor part of the dose and were not common to either urine or faeces. A summary of the distribution of the parent compound and metabolites in urine and faeces is provided in Table 5.1.3-08.

Table 5.1.3-08: Amounts of metabolites in urine and faeces after a single oral dose of 2 mg/kg [ethyl-1-^{14}C]BYI 02960. The data are given in % of the dose

Peak no.	(BYI 02960-)	Urine	Faeces	Total
1	DFA	5.28	0.49	5.77
6	difluoroethyl-amino-furanone	3.63	---	3.63
7	OH-gluA (isomer 1)	1.40	---	1.40
8	OH-gluA (isomer 3)	1.79	---	1.79
6	difluoroethyl-amino-furanone	3.63	---	3.63
7	OH-gluA (isomer 1)	1.40	---	1.40
8	OH-gluA (isomer 3)	1.79	---	1.79
11	OH	16.13	7.60	23.73
12	parent	51.96	3.79	55.75
13	iso-OH	---	0.43	0.43
	Identified	86.19	12.31	92.50
2	unknown 1	0.64	---	0.64
3	unknown 2	0.68	---	0.68
4	unknown 3	0.36	---	0.36
5	unknown 4	0.37	---	0.37
9	unknown 5	---	0.10	0.10
10	unknown 6	---	0.17	0.17
	Characterized	2.04	0.27	2.31
	Total in extracts	82.23	12.58	94.81
Total in urine and faeces		**82.23**	**13.51**	**95.74**

I. Biotransformation pathway

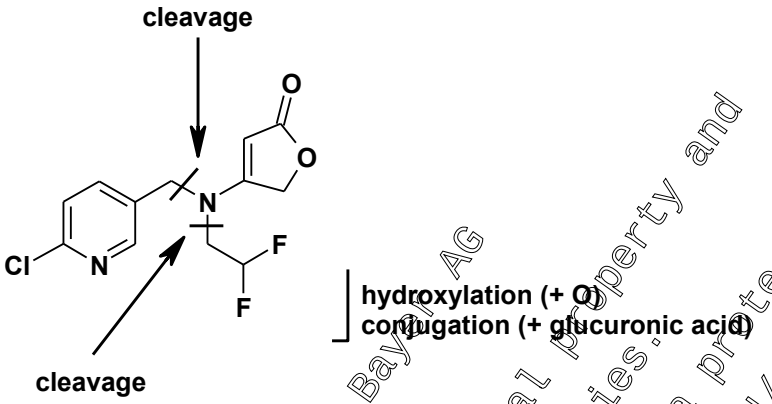
The main metabolic reactions of [ethyl-1-^{14}C]BYI 02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid,
- cleavage of the difluoroethyl group leading to BYI 02960-DFA and
- cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

Figure 5.1.3-04 schematically shows the positions in the molecule, which are involved in these metabolic reactions.

The proposed biotransformation pathway of [ethyl-1-^{14}C] BYI 02960 is presented in Figure 5.1.3-05.

Figure 5.1.3-04: Positions involved in metabolic degradation of BYI 02960

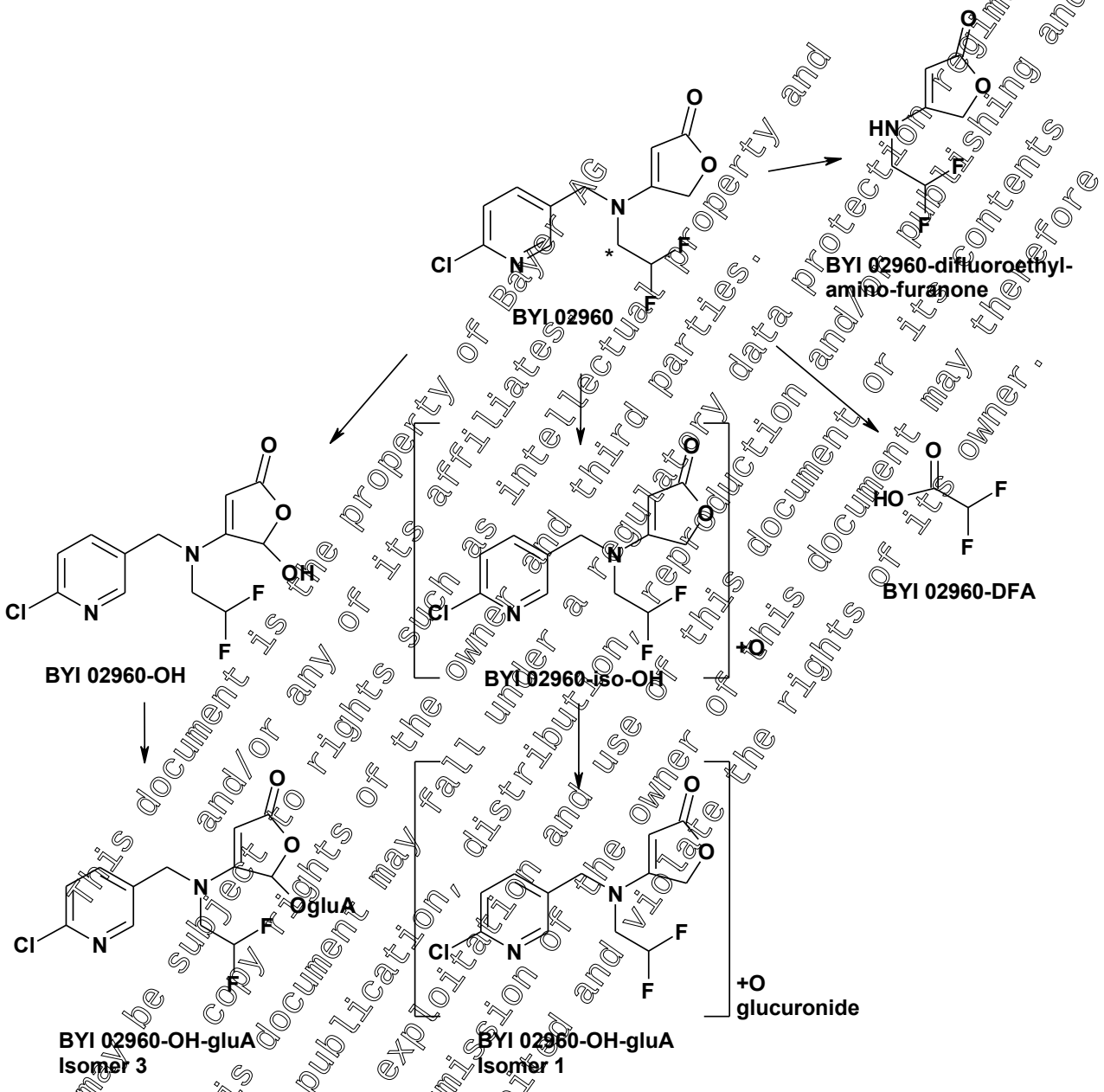


III. Conclusions

The kinetic and metabolic behavior of [ethyl-1-^{14}C]BYI 02960 in male Wistar rats is characterized as follows:

- The test compound was almost completely absorbed; more than 85% of the administered dose was detected in the urine and the body without GIT at sacrifice.
- The distribution of the radioactivity in the body was fast and the maximum plasma level (C_{max}) was reached within one hour after administration. The radioactivity concentration declined slowly to ca. 50% of C_{max} after 8 hours and to ca. 8% of C_{max} at sacrifice.
- Excretion of radioactivity was fast and mainly renal. The major part of the dose (>87%) was excreted within 24 hours after treatment, but continued until sacrifice. Particularly, the major part of BYI 02960-DFA was excreted on days 2 and 3.
- At sacrifice, a small proportion of ca. 3% of the dose was detected in the body without GIT. The residue concentration in plasma was 0.158 mg/kg, for most other organs and tissues levels were in the range between 0.05 and 0.1 mg/kg.
- Parent compound, one major and five minor metabolites were identified in all samples. Identification rates were >95% of the total radioactivity in urine and >85% of the total radioactivity in faeces.
- Approx. 92% of the dose was identified in excreta. Another ca. 3% of the dose corresponding to 7 unknown metabolites was characterized by their chromatographic behavior. All metabolites representing more than 1% of the administered dose were identified.
- The metabolic transformation of BYI 02960 was principally oxidative in nature and took place at 3 different positions of molecule.
- The metabolic pattern was in good accordance with that obtained from the corresponding organ metabolism rat study (KIIA 5.1.3/02).

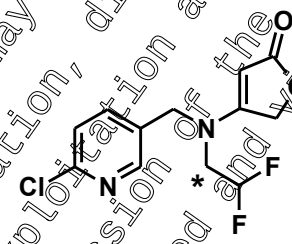
Figure 5.1.3.05: Proposed metabolic pathway of [ethyl-1-^{14}C]BYI 02960 in male rats



Report:	**KIIA 5.1.3/02, [redacted]; 2011**
Title:	[Ethyl-1-^{14}C]BYI 02960 – Metabolism in Organs and Tissues of Male and Female Rats (3 time-points)
Report No & Document No	MEF 11/270 M-415416-02-1
Guidelines:	OECD Guideline for Testing Chemicals, 417: Toxicokinetics US EPA Health Effects Test Guidelines OPPTS 870.7485, Metabolism and Pharmacokinetics Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	Yes, fully compliant US EPA – FIFRA Good Laboratory Practice (40 CFR Part 160) Principles of Good Laboratory Practice – German Chemical Law (Chemikaliengesetz), dated 2002-06-20, current version of Annex 1 JAPAN MAFF - Notification on the Good Laboratory Practice Standards for Agricultural Chemicals (11 Nousan 6283) notified 1999-10-01
Testing Facility and Dates	[redacted] Experimental work: 2010-11-5 - 2011-03-14

Executive Summary

The depletion of radioactive residues from plasma as well as organs and tissues, the excretion with urine and metabolism of the new insecticide BYI 02960 (common name: flupyradifurone) were investigated in male and female Wistar rats. Three groups of 4 male and 4 female rats each were administered orally by gavage with a single dose of BYI 02960 in 0.5% aqueous Tragacanth® at a dose level of 3 mg/kg bw. The test compound was labelled with ^{14}C in the C1-position of the ethyl side chain of the molecule as shown below:



***: denotes the ^{14}C-label position**

The animals were sacrificed 1 h, 6 h and 24 h after dosing. The total radioactivity was determined in urine for the time periods 0 - 1 h, 0 - 6 h and 0 - 24 h as well as in plasma, liver, kidney, muscle (leg) and fat (perirenal) at sacrifice. The metabolism was investigated in urine and plasma, as well as in extracts of liver, kidney, muscle, and fat.

The mean recovery for male rats ranged from 95.9 to 98.0% and for female rats from 100.3 to 103.7% of the given dose. The entire balances for the total radioactivity detected in urine, the combined GIT and faeces sample, skin, organs and tissues at sacrifice are shown in Table 5.1.3-09:

Table 5.1.3-09: Recovery of radioactivity in urine, plasma and organs following a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. Data are provided in % of the given dose

	Male			Female		
Test period [h]	**1**	**6**	**24**	**1**	**6**	**24**
Urine	6.22	36.53	71.80	8.76	39.70	85.88
Plasma	1.09	0.62	0.21	1.27	0.61	0.13
Carcass	38.32	24.47	4.25	51.19	29.57	3.08
Kidneys	1.30	0.73	0.08	1.17	0.63	0.06
Liver	6.80	3.44	0.60	7.30	3.62	0.39
GIT + faeces	27.24	21.18	16.61	13.94	14.26	9.44
Skin	14.64	9.19	2.13	17.54	10.48	1.45
Fat	0.10	0.04	0.01	0.15	0.09	0.01
Muscle	2.31	1.14	0.17	2.34	1.35	0.13
Balance	**98.03**	**97.33**	**95.85**	**103.70**	**100.30**	**100.60**

The renal excretion commenced immediately after administration and increased until 24 h to 71.8% of the dose in male and to 85.9% in female rats.
The highest radioactivity concentrations were detected in the organs and tissues as well as in the combined GIT and faeces 1 h after administration. The distribution of the radioactivity within the central compartments of the body (blood, liver, and kidney) was fast and showed a distinctive preference towards liver and kidney as the main metabolic and excretory organs. As shown in Table 5.1.3.10, all values decreased significantly until 24 h after administration. It is expected that the remaining radioactivity is further eliminated. There are no indications of irreversible binding or retention of radioactivity in organs and tissues.

Table 5.1.3.10: Equivalent concentration of radioactivity in urine, plasma and organs following a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960.
Data are provided as equivalent concentrations (mg/kg tissue)

	Male			Female		
Test period [h]	**1**	**6**	**24**	**1**	**6**	**24**
Plasma	2.166	1.494	0.491	2.728	1.661	0.343
Kidneys	4.747	2.896	0.317	4.901	2.795	0.267
Liver	4.281	2.507	0.367	5.770	3.225	0.243
Skin	2.003	1.271	0.294	2.398	1.470	0.203
Fat	0.911	0.493	0.108	1.079	0.655	0.047
Muscle	2.336	1.352	0.208	2.902	1.682	0.167

For the investigation of metabolism the pooled urine and plasma samples were analysed without further purification or extraction. Liver, kidney, muscle and fat samples were extracted using conventional methods. The extraction yield ranged from 88% to 99% of the total radioactivity.
BYI 02960 was intensively metabolized. Metabolic reactions took place at least at 3 different positions of the molecule. The majority of components were identified (ca. 97 - 100% of the radioactivity in plasma and in extracts of organs and tissues as well as ≥ 95% of the radioactivity in urine.

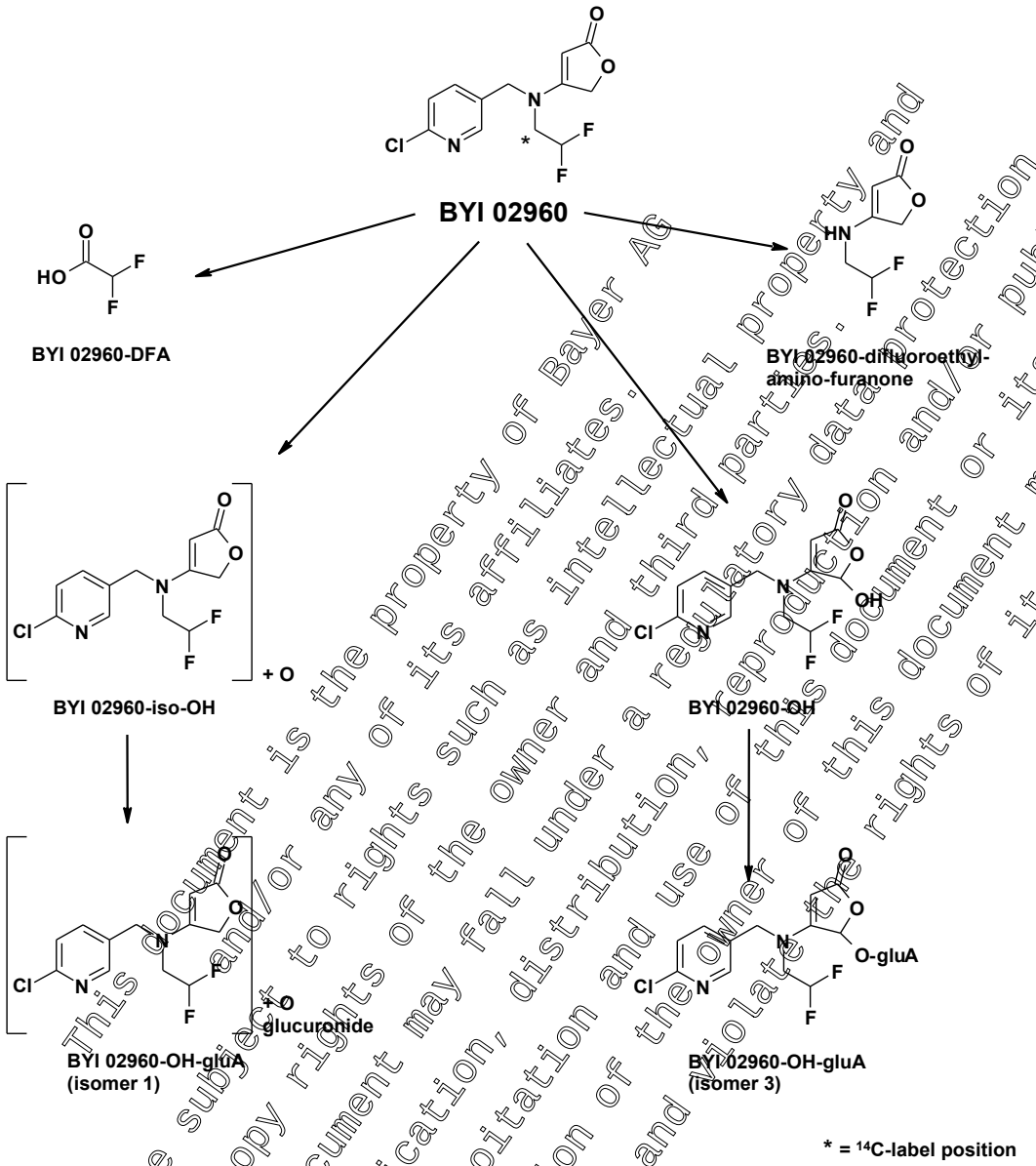
In the 24 hours samples of plasma, and organs and tissues BYI 02960-DFA was the by far largest metabolite accounting for more than 50% of the radioactivity. For all other identified metabolites, the values were below 10%. The contribution of the parent compound in these samples ranged from 6 to 38% of the radioactivity. In the respective urine sample, the parent compound was the largest radioactive component (approx. 48% of the dose in males and 77% in females).
The metabolism was qualitatively similar in male and female rats. However, there were quantitative differences because the degradation of the parent compound was significantly higher in male as compared to female rats.
The principal metabolic reactions of [ethyl-1-^{14}C]BYI 02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid,
- cleavage of the difluoroethyl group leading to BYI 02960-DFA and
- cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

The metabolism results are in good accordance with those obtained from the ADME rat study which was conducted with the [ethyl-1-^{14}C] radiolabelled test compound (KIIA 5.1.3/01).

The proposed metabolic pathway is shown in Figure 5.1.3-06.

Figure 5.1.3-06: Proposed metabolic pathway of [ethyl-1-^{14}C]BYI 02960 in male and female rats



I. Material and Methods

A. Material

1. Test Material:

IUPAC Name:	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name:	BYI 02960
Common name:	Flupyradifurone
Empirical formula:	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass:	288.68 g/mol
Water solubility:	pH 4 = 3.2 mg/L, pH 7 = 3.2 mg/L pH 9 = 3.0 mg/L
n-Octanol/water partition coefficient:	pH 4 = 1.2, pH 7 = 1.2, pH 9 = 1.2
Labelling:	[ethyl-1-^{14}C]
Specific radioactivity of radiolabelled batches:	3.93 MBq/mg = 2.36 x 10^5 dpm/µg = 106.28 µCi/mg = 30.68 Ci/mol
Specific radioactivity used for administration:	3.93 MBq/mg = 2.36 x 10^5 dpm/µg = 106.28 µCi/mg = 30.68 Ci/mol
Radiochemical purity:	> 99 % (certified, HPLC with radiodetection) > 98 % (TLC with radiodetection)
Dose level:	3 mg/kg body weight
Vehicle:	0.5 % aqueous tragacanth suspension
Stability of the test material:	The stability of [ethyl-1-^{14}C]BYI 02960 was determined by radio-HPLC of the administration suspensions immediately after dosing

2. Test Animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	Wistar Hsd/Cpb: WU
Breeding facility:	[redacted]
Sex and numbers involved:	12 male and 12 female animals
Age:	Males: ca. 6 weeks at the time of delivery Females: ca. 8 weeks at the time of delivery
Body weight:	191 - 209 g at the time of administration
Acclimatization:	Makrolon® cages on wood shavings in the test facility for 7 days prior to the administration
Identification:	Cage cards on which the study number, test compound name and individual animal number were displayed. Additional labelling with water-insoluble spots on the tail
Housing:	After administration of the radiolabelled test compound individually in Makrolon® metabolism cages under conventional hygienic conditions in air-conditioned rooms Temperature 22 - 25 °C, relative humidity 54 - 77 %.

	12/12 hours light / dark cycle, air change 10 - 15 times per hour
Feed and water:	Rat/mice maintenance long life diet (no. 3883.0.15), supplied by Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland (ca. 16 g per animal and day) Last feeding ca. 16 h prior to dosing Next feeding ca. 6 h after dosing Tap water from municipal water supply, *ad libitum*

B. Study Design

1. Dosing

Each rat received a single oral dose of 3 mg/kg bw [ethyl-1-^{14}C]BYI 02960 suspended in 0.5 % aqueous tragacanth after ca. 16 h of fasting. The dosing suspension (0.3 mg/mL) was prepared in a cold room at 5°C.

The suspension was administered by gavage using a syringe attached to an animal-feeding knob cannula. Each animal received a volume of 2 mL (10 mL/kg bw calculated for a nominal body weight of 200 g). The individual amounts of the administered test substance were calculated by dividing the amount of the administered radioactivity by the specific radioactivity. The actual mean administered dose of [ethyl-1-^{14}C]BYI 02960 was 3.1 mg/kg. The stability of the test compound in the suspension was assured by radio-HPLC analysis after administration.

2. Collection of excreta

After administration the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine and faeces. Urine was collected separately for each animal in a cryogenic trap cooled with dry ice from 0 - 1 h, 0 - 6 h and 0 - 24 h. The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction. The radioactivity was determined by LSC. Faeces were collected from 0 - 1 h, 0 - 6 h and 0 - 24 h after dosing separately for each animal in a cryogenic trap cooled with dry ice. The radioactivity of these individual faeces samples was not determined, because they were added at sacrifice to the gastrointestinal tract (GIT) of the corresponding rat.

3. Plasma, and organs and tissues at sacrifice

At sacrifice (1 h, 6 h, 24 h), the individual blood samples were collected in heparinized test tubes and separated into plasma and blood cells by centrifugation. After weighing, aliquots were taken for determination of radioactivity by LSC.

The dissected tissue samples (GIT including faeces, skin, and carcass including sedimented blood cells) were transferred into plastic vessels for recording their individual fresh weights. The combined GIT/faeces-sample and an aliquot of depilated skin were lyophilised. After weighing and homogenization, aliquots were taken for determination of total radioactivity by combustion/LSC.

The whole carcass and blood cells samples were passed several times through a mincing machine in half-frozen state. From this tissue pulp, an aliquot was lyophilised, homogenized and weighed, before aliquots were taken for determination of the radioactivity by combustion/LSC.

Liver, kidneys, muscle and fat were weighed separately after collection. In order to obtain sufficient sample material for extraction of radioactive residues and metabolic profiling, the total radioactivity of

the individual organs and tissue samples was not determined. Instead, respective sample pools were generated for each test group. The mean dpm/g-values which were used for further calculations were derived from the sum of extracts and solids of the respective samples after extraction.

4. Sacrifice

The rats were sacrificed in Pentobarbital-Na anaesthesia (Narcoren®, supplied by [redacted], Germany) by transection of the cervical vessels and exsanguinated.

5. Measurement of radioactivity

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). All solid samples were combusted in an oxygen atmosphere using an oxidizer. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

6. Analytical methods

Samples were analyzed by radiochromatographic (HPLC, TLC) and spectroscopic (LC-MS, ^{1}H-NMR) methods.

7. High Performance Liquid Chromatography (HPLC)

HPLC-radioactivity analysis was performed on an Agilent 1100 system with radiometric- and UV-detection. The separation was carried out on a reversed phase column using a neutral water / acetonitrile gradient. Detailed information of the HPLC-methods and instruments set-up can be found in the report. All solvents were of HPLC-quality.

The chromatograms were recorded and quantitatively evaluated using the software package GINA® (raytest, Benzstraße 4, D-75334 Straubenhardt, Germany). The ^{14}C-trace of a chromatogram was divided into regions of interest (ROI's), according to the separated radioactive peaks. Additionally, background regions (BKG's) were defined and used for background correction. The background corrected area counts from all regions of interest were used for calculation of the quantitative distribution of the individual components in the sample. A quantifiable radioactive peak was regarded relevant if resulting in a signal approx. 2.5 times above the background noise.

For HPLC co-chromatography, an aliquot of the sample was mixed with the reference compound before injection. The detection was carried out either by UV-absorbance of the non-radiolabelled or by ^{14}C-detection of the radiolabelled reference compound. The time delay between the radioactivity and UV-absorbance detectors was compensated by a parameter set in the software. Chromatographic correspondence with the non-radiolabelled reference compound was assessed by comparison of the UV-trace and the associated ^{14}C-trace. Matching with the radiolabelled reference compound was assessed by comparison of the ^{14}C-chromatogram of the mixture with the ^{14}C-chromatogram of the sample without the reference compound.

8. Thin Layer Chromatography (TLC)

For Thin Layer chromatography the samples were applied to the TLC-plates (silica gel 60 F_{254}, normal phase) as a thin even band horizontally to and just above the solvent level using a Linomat IV - instrument (Camag, Berlin, Germany). The plates were developed using ethyl-acetate / isopropanol / water / acetic acid (65 / 24 / 11 / 1; v/v) as a solvent system.

The TLC-bands or spots were visualized under a UV-light (254 nm). The radioactive zones were detected using a Fujibas® 2000 bio imaging system (Fuji, Japan & raytest, Straubenhard, Germany). Fujibas® imaging plates were exposed to the TLC-plates. The imaging data were identified with Basreader software (version 2.13e, raytest, Straubenhard, Germany. Evaluation and visualization of recorded data was performed with AIDA software (raytest, Straubenhard, Germany). The detection limit (LOD) was 5 - 10 dpm/zone for ^{14}C after an exposure of at least 14 hours.
TLC co-chromatography was carried out by mixing an aliquot of the sample solution with the radiolabelled reference compound solution before the mixture was applied to the TLC-plate. As second method, an aliquot of the sample solution was applied to the TLC-plate as a 1 to 1.5 cm-wide band. The reference compound solution was also applied as a 1 to 1.5 cm-wide band, part of which (ca. 5 to 8 mm) overlapped with the band of the sample solution. After development of the TLC plate, chromatographic correspondence of radiolabelled components of the sample solution with the reference compound was assessed by analysis of the individual radioluminograms.

9. Mass spectroscopy
Electro-spray ionization MS spectra (ESI) were obtained either with a TSQ 7000 instrument or with a LTQ Orbitrap XL mass spectrometer (Thermo or Finnigan respectively, San Jose, CA, U.S.A.). The HPLC instrument used for chromatography was an Agilent HP1100 (Agilent, Waldbronn, Germany). The eluate of the HPLC-column was split between the UV-detector followed by a radioactivity detector (Ramona Star, Raytest, Straubenhardt, Germany) and the MS spectrometer.

10. ^{1}H-NMR spectroscopy
600 MHz ^{1}H-NMR-spectra were recorded using a BRUKER AV 600 instrument (Bruker, Karlsruhe, Germany).

11. Preparation of samples, extraction and analysis
Urine
The urine samples from all four animals of each test were combined. The amount of radioactivity applied for analysis was calculated from the in-life data of the respective samples. Aliquots thereof were taken without further sample processing for metabolite profiling by HPLC.

Plasma
The plasma samples from all four animals of each test were combined. The amount of radioactivity applied for analysis was calculated from the in-life data of the respective samples. Aliquots thereof were taken without further sample processing for metabolite profiling by HPLC.

Liver, kidney and muscle
The organ samples from all four animals of each test were combined for extraction. Consecutive solvent extractions were performed by macerating the samples 3-times with ACN/water (8/2, v/v) using a Polytron (model PT 3100) homogenisator (ca. 3 min. at ca. 16,000 rpm). At each step, the respective sample was separated by filtration into extract and solids. The volume of each extract was measured and the radioactivity of an aliquot was determined by LSC. Aliquots were taken from the remaining final solids for radioactivity measurement by combustion/LSC. Those extracts were combined for which the radioactivity count of the respective aliquot was higher than 20 dpm.

A Phenomenex Strata C18E SPE-cartridge was conditioned successively with an equal volume of methanol, water and ACN/water (8/2, v/v) before use. The combined organ extract was sucked slowly through the cartridge to remove the lipid fraction of the matrix. Unbound material was removed by washing the column with ACN/water (8/2, v/v). The percolate and rinse samples were combined, the volume was determined and aliquots were taken for radioactivity measurement. Possible bound material was removed by washing the column finally with DCM/MeOH (1/1, v/v). After determination of the volume, aliquots from each sample were taken for radioactivity measurement. The percolate and rinse samples were concentrated after addition of an emulsifier (200 - 500 µL). The pH-value was adjusted to ca. 7. Aliquots of this solution were taken for radioactivity measurement and metabolite profiling by HPLC.

Fat

The fat samples from all four animals of each test were combined for extraction. Consecutive solvent extractions were performed by macerating the sample at first once with a 1 / 1-mixture of n-heptane and ACN/water (8/2, v/v) and afterwards 2-times with ACN/water (8/2, v/v) using a Polytron (model PT 3100) homogenisator (ca. 3 min. at ca. 16,000 rpm). At each step, the respective sample was separated by filtration into extract and solids. The filtrate from the first extraction step was transferred to a separator funnel in order to separate the n-heptane from the ACN/water phase. The volume of each extract/phase was measured and the radioactivity of an aliquot was determined. Aliquots from the remaining final solids were taken for radioactivity measurement by combustion/LSC. Those ACN/water-extracts were combined for which the radioactivity count of the respective aliquot was higher than 20 dpm.

A Phenomenex Strata C18E SPE-cartridge was conditioned successively with an equal volume of methanol, water and ACN/water (8/2, v/v) before use. The combined fat extract was sucked slowly through the cartridge to remove the lipid fraction of the matrix. Unbound material was removed by washing the column with ACN/water (8/2, v/v). The percolate and rinse samples were combined, the volume was determined and aliquots were taken for radioactivity measurement. Possible bound material was removed by washing the column finally with DCM/MeOH (1/1, v/v). Following determination of the volume, aliquots from each sample were taken for radioactivity measurement. The percolate and rinse samples were concentrated after addition of an emulsifier (200 - 500 µL). The pH-value was adjusted to ca. 7. Aliquots of this solution were taken for radioactivity measurement and metabolite profiling HPLC.

12. Identification / characterization and quantification of residues

The identification of parent compound and metabolites was mainly based on high resolution LC-MS analysis, HPLC- and TLC co-chromatography using authentic reference compounds and chromatographic comparison. All chromatograms were integrated for quantification. Details of the procedure for identification, characterization and quantification of residues are provided in the report.

II. Results and Discussion

A. Recovery and renal excretion

The detailed recovery rates of radioactivity in urine, organs and tissues, skin and the combined GIT and faeces sample are shown in Table 5.1.3-11. The mean recoveries for male rats ranged from 95.85% to 98.03% and for female rats from 100.30% to 103.70% of the given dose.
The renal excretion commenced immediately after administration and increased until 24 hours to 71.8% of the given dose in male and 85.9% in female rats (Table 5.1.3-11). These results are in very good accordance to the urinary excretion behavior of all other rat studies.

Table 5.1.3.11: Recovery of radioactivity in urine, plasma and organs following a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. Data are provided as % of the given dose

	Male			Female		
Test period [h]	**1**	**6**	**24**	**1**	**6**	**24**
Urine	6.22	36.53	71.80	8.76	39.70	85.88
Plasma	1.09	0.62	0.21	1.27	0.61	0.13
Carcass	38.32	24.47	4.25	51.19	29.57	3.08
Kidneys	1.30	0.73	0.08	1.17	0.63	0.06
Liver	6.80	3.44	0.60	7.30	3.62	0.39
GIT + faeces	27.24	21.18	16.61	13.94	14.26	9.44
Skin	14.64	9.19	2.13	17.54	10.48	1.45
Fat	0.10	0.04	0.01	0.15	0.09	0.01
Muscle	2.31	1.14	0.17	2.34	1.35	0.13
Balance	**98.03**	**97.33**	**95.85**	**103.70**	**100.30**	**100.60**

B. Radioactive Residues in Plasma and in Organs and Tissues

In male rats, sacrificed at 1 hour after administration, approx. 64.6% of the dose was detected in organs and tissues and approx. 27.2% in the GIT and faeces sample. After 24 hours, the value for organs and tissues declined to approx. 7.5%. These values indicated a fast distribution of the absorbed radioactivity within the body followed by a quick elimination finally leading to a significant increase of the urinary excretion from ca. 6.2% to ca. 71.8%. The highest radioactivity concentrations were detected at the initial time-point. As shown in Table 5.1.3-12, all values declined significantly until the end of test.
The situation was slightly different in female rats. After sacrifice at 1 hour h after administration, approx. 81% of the dose was detected in organs and tissues and approx. 13.9% in the GIT and faeces sample. After 24 hours, the value of organs and tissues declined to approx. 5.3%. These values indicated again a fast distribution of the absorbed radioactivity within the body followed by a quick elimination finally leading to a significant increase of the urinary excretion from approx. 8.8% to approx. 85.9%. Similar to male rats, the highest radiooactivity concentrations were detected in the organs and tissues at the initial time-point. As shown in Table 5.1.3-12, all values significantly declined until the end of test. In both cases, it is expected that the remaining radioactivity is further eliminated. There are no indications of irreversible binding or retention of radioactivity in organs and tissues.

Table 5.1.3.12: Radioactive residues in urine, plasma and organs following a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. Data are provided as equivalent concentrations (mg/kg tissue)

	Male		Female	
Test period [h]	1	24	1	24
Plasma	2.166	0.491	2.728	0.343
Kidneys	4.747	0.317	4.901	0.267
Liver	4.281	0.367	5.770	0.243
Skin	2.003	0.294	2.398	0.203
Fat	0.911	0.108	1.079	0.047
Muscle	2.336	0.208	2.902	0.167

C. Extraction efficiency of residues

Sample pools of liver, kidney, muscle, and fat were extracted using conventional methods. The resulting extracts represented between approx. 88% and more than 99% of the total radioactivity after purification using reversed phase SPE. A summary of the extraction efficiency is shown in Table 5.1.3-13.

Table 5.1.3-13: Extraction efficiency (% of TRR) of liver, kidney, muscle and fat samples of male and female rats sacrificed 1 h, 6 h, and 24 h after a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960

	Sacrifice	Liver	Kidney	Muscle	Fat
Male	1 h	99.5	99.8	100.0	100.0
	6 h	97.4	99.2	99.8	99.9
	24 h	88.1	95.6	99.0	96.8 + 2.6 *
Female	1 h	99.8	99.9	100.0	100.0
	6 h	99.5	99.7	99.9	99.9
	24 h	95.3	96.3	99.3	98.2 + 1.2 *

* : Heptane-phase

D. Identification / Characterisation and quantification of residues

The following strategy was used for identification of parent compound and metabolites:
Spectroscopic investigations (LC-MS) were conducted on the 0 - 24 h urine samples of male rats and on the 6-hour plasma sample of male rats. An HPLC-comparison of the 0 - 6 h urine sample of male rats with the radiolabelled reference compounds BYI 02960-difluoroethyl-amino-furanone and the isomers 1 and 3 of BYI 02960-OH-gluA was carried out. TLC co-chromatography was applied for urine and plasma samples, as well as extracts from liver, kidney and muscle of male rats (0 - 24h) with the radiolabelled reference compound BYI 02960-DFA. Furthermore, a comparison of the HPLC-radiochromatograms from all samples of each individual test was made.
Other peaks or peak groups additionally detected in the HPLC-profiles were designated as "unknown". They were characterized by their behavior during extraction and clean-up and the retention times in the HPLC-chromatograms. Parent compound and metabolites were quantified in the original urine and

plasma samples and in the conventional acetonitrile/water extracts of organs and tissues by integration of the ^{14}C-signals in the HPLC-chromatograms.

1. Metabolites in urine

In the urine of male rats (0 - 24 h) the major metabolites with more than 1% but less than 5% of the dose were identified as BYI 02960-OH-gluA (isomer 1 and 3), BYI 02960-DFA and BYI 02960-difluoroethyl-amino-furanone. The most prominent metabolite BYI 02960-OH accounted for approx. 12.3% of the dose. By far the largest component was identified as parent compound (approx. 47.7% of the dose). The identification rate amounted to 95.1%.

In the urine of female rats (0 - 24 h) the only metabolite accounting for more than 1% but less than 5% of the dose was identified as BYI 02960-DFA. The main metabolite BYI 02960-OH accounted for approx. 6.4% of the dose. As with male rats, by far the largest component in the urine was identified as parent compound (approx. 76.5% of the dose). The identification rate amounted to 99.5% of radioactivity. The detailed results of the metabolic profiles in urine are summarized in Table 5.1.3-14.

Table 5.1.3-14: Quantification of parent compound and metabolites in urine of male and female rats sacrificed 1 h, 6 h, and 24 h after a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. Data are presented as % of the given dose

	Male			Female		
Sampling period	**0 - 1 h**	**0 - 6 h**	**0 - 24 h**	**0 - 1 h**	**0 - 6 h**	**0 - 24 h**
Parent compound	4.96	25.48	47.69	8.19	36.15	76.48
DFA	---	0.22	1.91	---	0.11	1.70
unknown	---	0.38	0.72	---	0.18	0.42
unknown	---	0.15	0.35	---	---	---
unknown	---	0.30	0.60	---	---	---
unknown	---	---	0.43	---	---	---
unknown	---	---	0.27	---	---	---
BYI 02960-difluoroethyl-amino-furanone	0.12	1.55	3.09	0.07	0.33	0.87
BYI 02960-OH-gluA (isomer 1)	0.12	0.66	1.55	---	0.10	---
unknown	---	---	0.46	---	0.14	---
unknown	---	---	---	---	0.38	---
BYI 02960-OH-gluA (isomer 3)	0.13	0.74	1.72	---	0.08	---
unknown	0.08	---	0.34	---	---	---
unknown	---	0.07	0.10	---	---	---
unknown	---	0.15	0.18	---	---	---
BYI 02960-OH	0.82	6.85	12.33	0.51	2.24	6.42
Total identified	6.15	35.49	68.28	8.76	39.00	85.46
Total characterized *	0.08	1.04	3.52	---	0.70	0.42
Sum total	**6.22**	**36.53**	**71.80**	**8.76**	**39.70**	**85.88**
Identification rate	**98.8%**	**97.2%**	**95.1%**	**100.0%**	**98.2%**	**99.5%**

*: Peaks were characterized based on their retention time in HPLC-analysis:

2. Metabolites in Plasma

Male rats

In the 1 hour sample, three metabolites with concentrations of about 0.05 to 0.06 mg/kg each were identified as BYI 02960-DFA, BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH. The parent compound was the dominating component in the plasma (1.998 mg/kg). At 6 hours after administration, the concentrations for BYI 02960-DFA and BYI 02960-difluoroethyl-amino-furanone had increased by factors of 6 and 1.6, respectively. For BYI 02960-OH and the parent compound on the other hand, a decline by factors of 1.3 and 2 was observed. Two minor unknown metabolites with concentrations of less than 0.03 mg/kg were additionally detected. The concentration of BYI 02960-DFA at 24 hours after administration had further increased by a factor of 1.3 whereas a sharp decline by a factor of 31 was determined for the parent compound. The concentrations for all other metabolites were below 0.01 mg/kg. The identification rates ranged from 97 to 100% of the total radioactivity.

Female rats

BYI 02960-DFA was the only metabolite detected in all plasma samples showing increasing concentrations from 0.057 mg/kg at 1 hour to 0.280 mg/kg at 24 hours after dosing. Accordingly the concentration of the parent compound declined from 2.671 to 0.063 mg/kg (factor 42). The identification rates amounted to 100% of the total radioactivity. The detailed results of the metabolic profiles in plasma are summarized in Table 5.1.3-15.

Table 5.1.3-15: Concentration of parent compound and metabolites in plasma of male and female rats sacrificed 1 h, 6 h, and 24 h after a single oral dose following a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. Data are presented as mg/kg

	Male			Female		
Sampling time	**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Parent compound	1.998	0.976	0.031	2.671	1.486	0.063
BYI 02960-DFA	0.058	0.348	0.448	0.057	0.175	0.280
unknown	---	0.024	---	---	---	---
BYI 02960-difluoroethyl-amino-furanone	0.052	0.084	0.005	---	---	---
unknown	---	0.018	---	---	---	---
BYI 02960-OH	0.058	0.045	0.007	---	---	---
Total identified	2.166	1.452	0.491	2.728	1.661	0.343
Total characterized *	---	0.042	---	---	---	---
Sum total**	**2.166**	**1.494**	**0.491**	**2.728**	**1.661**	**0.343**

* : Peaks were characterized based on their retention time in HPLC-analysis
** : Sum total = total identified or characterized + solids (unextractable) + samples not analyzed

3. Metabolites in liver

Male rats

The major metabolites of the 1 hour sample with residue-concentrations in a range of 0.049 to 0.187 mg/kg were identified as BYI 02960-DFA, BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH. The parent compound was the largest component detected in the liver extract (3.9 mg/kg). At 6 hours after administration, the concentrations for BYI 02960-DFA and BYI 02960-difluoroethyl-amino-

furanone had increased by factors of 3.7 and 1.2, respectively. For BYI 02960-OH and the parent compound on the other hand, a decline by factors of 1.2 and 2 was observed. Six minor metabolites with concentrations below 0.05 mg/kg were additionally detected from which one was identified as BYI 02960-OH-gluA (isomer 1). The concentration of BYI 02960-DFA at 24 hours after administration had further increased by a factor of 1.2 whereas a sharp decline by factors of 23 and 11 was determined for the parent compound and BYI 02960-OH. The identification rates ranged from 86 to 98% of the total radiuoactivity.

Female rats

BYI 02960-OH was the only major metabolite of the 1 hour sample with residue-concentrations of more than to 0.05 mg/kg. Six minor metabolites with concentrations below 0.05 mg/kg were detected and from these BYI 02960-DFA and BYI 02960-difluoroethyl-amino-furanone were identified. The parent compound was the largest component detected in the liver extract (5.519 mg/kg). At 6 hours after administration, the concentration of BYI 02960-DFA had increased by a factor of 3.5. For BYI 02960-OH and the parent compound on the other hand, a decline by factors of 1.7 and 1.9 was observed. Three minor metabolites with concentrations below 0.05 mg/kg were additionally detected one of which was identified as BYI 02960-difluoroethyl-amino-furanone. The concentration of BYI 02960-DFA at 24 hours after administration had further increased by a factor of 1.2 whereas a sharp decline by factors of 35 and 13 was determined for the parent compound and BYI 0960-OH, respectively. The identification rates ranged from 92 to 99% of the total radioactivity.

The detailed results of the metabolic profiles in the liver are summarized in Table 5.1.3-16.

Table 5.1.3.16: Concentration of parent compound and metabolites in the liver of male and female rats sacrificed 1 h, 6 h, and 24 h after a single oral dose following a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. Data are presented as mg/kg

	Male			Female		
Sampling time	**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Parent compound	3.900	1.903	0.084	5.519	2.977	0.084
BYI 02960-DFA	0.049	0.183	0.218	0.033	0.116	0.136
unknown	---	0.012	---	0.019	---	---
unknown	---	0.012	---	---	---	---
BYI 02960-difluoroethyl-amino-furanone	0.070	0.086	---	0.022	0.018	---
BYI 02960-OH-gluA (isomer 1)	---	0.021	---	---	---	---
unknown	---	0.013	---	---	---	---
unknown	---	0.013	---	0.017	0.016	---
unknown	---	---	---	---	---	0.002
unknown	---	---	---	---	---	0.002
unknown	---	---	---	0.014	---	---
unknown	0.042	0.031	---	0.021	0.010	---
BYI 02960-OH	0.187	0.150	0.014	0.106	0.063	0.005
Total identified	4.296	2.343	0.316	5.696	3.190	0.225
Total characterized *	0.042	0.081	---	0.055	0.010	0.004
**Sum total ** **	**4.281**	**2.507**	**0.367**	**5.770**	**3.225**	**0.243**

* : Peaks were characterized based on their retention time in HPLC-analysis
** : Sum total = total identified or characterized + solids (unextractable) + samples not analyzed

4. Metabolites in kidney

Male rats

In the 1 hour sample, three metabolites with residue-concentrations in a range of 0.042 to 0.341 mg/kg were identified as BYI 02960-DFA, BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH. The parent compound was the largest component detected in the kidney extract (4.268 mg/kg). At 6 hours after administration, the concentrations for BYI 02960-DFA and BYI 02960-difluoroethyl-amino-furanone had increased by factors of 1.2 and 1.8, respectively. For BYI 02960-OH and the parent compound on the other hand, a decline by factors of 1.02 and 2 was observed. Three minor unknown metabolites with concentrations below 0.05 mg/kg were additionally detected. The concentration of BYI 02960-DFA at 24 hours after administration had further increased by a factor of 1.1 whereas a sharp decline by factors of 26 and 17 was determined for the parent compound and BYI 02960-OH. The identification rates ranged from 96 to 100% of the total radioactivity.

Female rats

The major metabolites of the 1 hour sample with residue-concentrations in a range of 0.038 to 0.155 were identified as BYI 02960-DFA and BYI 02960-OH. The parent compound was the dominating component detected in the kidney extract (4.695 mg/kg). At 6 hours after administration, the concentration for BYI 02960-DFA had increased by a factor of 3.5. For BYI 02960-OH and the parent compound on the other hand, a decline by factors of 1.3 and 9 was observed. The concentration of BYI 02960-DFA at 24 hours after administration had further increased by a factor of 1.1 whereas a sharp

decline by factors of 24 and 10 was determined for the parent compound and BYI 02960-OH, respectively. The identification rates ranged from 96 to 100% of the total radioactivity.
The detailed results of the metabolic profiles in the kidney are summarized in Table5.1.3-17.

Table 5.1.3-17: Concentration of parent compound and metabolites in the kidney of male and female rats sacrificed 1 h, 6 h, and 24 h after a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. Data are presented as mg/kg

	Male			Female		
Sampling time	**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Parent compound	4.268	2.108	0.082	4.695	2.530	0.102
BYI 02960-DFA	0.042	0.176	0.200	0.038	0.133	0.144
unknown	---	0.036	---	---	---	---
unknown	---	0.019	---	---	---	---
BYI 02960-difluoroethyl-amino-furanone	0.084	0.163	---	---	---	---
unknown	---	0.034	---	---	---	---
BYI 02960-OH	0.341	0.334	0.020	0.156	0.115	0.011
Total identified	4.735	2.772	0.303	4.888	2.778	0.257
Total characterized *	---	0.088	---	---	---	---
**Sum total ** **	**4.747**	**2.896**	**0.317**	**4.901**	**2.795**	**0.267**

* : Peaks were characterized based on their retention time in HPLC-analysis
** : Sum total = total identified or characterized + solids (unextractable) + samples not analyzed

5. Metabolites in muscle

Male rats

In the 1 hour sample, three metabolites of with residue-concentrations in a range of 0.021 to 0.061 mg/kg were identified as BYI 02960-DFA, BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH. The parent compound was the dominating component detected in the muscle extract (2.201 mg/kg). At 6 hours after administration, the concentrations for BYI 02960-DFA and BYI 02960-difluoroethyl-amino-furanone had increased by factors of 6.1 and 1.4, respectively. For BYI 02960-OH and the parent compound on the other hand, a decline by factors of 1.2 and 2 was observed. The concentration of BYI 02960-DFA at 24 hours after administration remained at the same level whereas a sharp decline by a factor of 15 was determined for the parent compound. BYI 02960-OH and BYI 02960-difluoroethyl-amino-furanone were not detected in this sample. The identification rates ranged from 95 to 100% of the total radioactivity.

Female rats

The major metabolites of the 1 hour sample with residue-concentrations in a range of 0.016 to 0.035 mg/kg were identified as BYI 02960-DFA, BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH. The parent compound was the dominating component detected in the muscle extract (2.827 mg/kg). At 6 hours after administration, the concentrations for BYI 02960-DFA and BYI 02960-difluoroethyl-amino-furanone had increased by factors of 3.0 and 1.2, respectively. For BYI 02960-OH and the parent compound on the other hand, a decline by factors of 1.7 and 1.8 was observed. The concentration of BYI 02960-DFA at 24 hours after administration had further increased by a factor of 1.5 whereas a sharp decline by factors of 29 and 7 was determined for the parent compound and BYI 02960-OH, respectively. The identification rates ranged from 99 to 100% of the total radioactivity.

The detailed results of the metabolic profiles in muscle are summarized in Table5.1.3-18.

Table 5.1.3-18: Concentration of parent compound and metabolites in muscle of male and female rats sacrificed 1 h, 6 h, and 24 h after a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960.
Data are presented as mg/kg

	Male			Female		
Sampling time	**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Parent compound	2.201	1.097	0.075	2.827	1.568	0.055
BYI 02960-DFA	0.021	0.129	0.124	0.023	0.070	0.108
BYI 02960-difluoroethyl-amino-furanone	0.050	0.070	---	0.016	0.019	---
BYI 02960-OH	0.061	0.049	---	0.035	0.021	0.003
Total identified	2.332	1.346	0.198	2.901	1.678	0.166
Sum total **	**2.336**	**1.352**	**0.208**	**2.902**	**1.682**	**0.167**

* : Peaks were characterized based on their retention time in HPLC-analysis
** : Sum total = total identified or characterized + solids (unextractable) + samples not analyzed

6. Metabolites in fat

Male rats

The parent compound was the only component detected in the 1 hour fat extract (0.911 mg/kg). At 6 hours after administration, three metabolites were found, two of them were identified as BYI 02960-DFA (0.051 mg/kg) and BYI 02960-OH (0.018 mg/kg). For the parent compound, a decline by a factor of 2.3 was observed The concentration of BYI 02960-DFA at 24 hours after administration had increased by a factor of 1.5 whereas a sharp decline by a factor of 13 was determined for the parent compound. BYI 02960-OH was not detected in this sample. The identification rates ranged from 93 to 100% of the total radioactivity.

Female rats

The parent compound was the only component detected in the 1 hour fat extract (1.079 mg/kg). At 6 hours after administration, BYI 02960-DFA was detected in the fat extract (0.031 mg/kg). For the parent compound, a decline by a factor of 1.7 was observed. The concentration of BYI 02960-DFA at 24 hours after administration remained stable whereas a sharp decline by a factor of 48 was determined for the parent compound. The identification rates ranged from 96 to 100% of the total radioactivity. The detailed results of the metabolic profiles in fat are summarized in Table 5.1.3-19.

Table 5.1.3.19: Concentration of parent compound and metabolites in fat of male and female rats sacrificed 1 h, 6 h, and 24 h after a single oral dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. Data are presented as mg/kg

	Male			Female		
Sampling time	**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Parent compound	0.911	0.389	0.030	1.079	0.624	0.013
BYI 02960-DFA	---	0.051	0.074	---	0.031	0.032
unknown	---	0.035	---	---	---	---
BYI 02960-OH	---	0.018	---	---	---	---
Total identified	0.911	0.458	0.104	1.079	0.654	0.045
Total characterized *	---	0.035	---	---	---	---
**Sum total ** **	**0.911**	**0.493**	**0.108**	**1.079**	**0.655**	**0.047**

* Peaks were characterized based on their retention time in HPLC-analysis
** Sum total = Total identified or characterized + solids (unextractable) + samples not analyzed

E. Comparison of the metabolic profiles

BYI 02960 was intensively metabolized. Metabolic reactions took place at least at 3 different structural positions of the molecule. The majority of the radioctive residues were identified (approx. 97 - 100% in plasma, and in extracts of organs and tissues as well as ≥ 95% of radioactivity in urine). In the 24 hours samples of plasma, and organs and tissues, BYI 02960-DFA was by far the dominating metabolite accounting for more than 50% of the radioactivity. All other identified metabolites contributed to less than 10%. The contribution of the parent compound in these samples ranged from 6 to 38% of the radioactivity. In urine, the parent compound was the dominating radioactive component (approx. 48% of the dose in males and 77% in females).
The metabolism was qualitatively similar in male and female rats but with quantitative differences. The degradation of the parent compound to the different metabolites in male rats was significantly higher than in female rats.

F. Biotransformation pathway

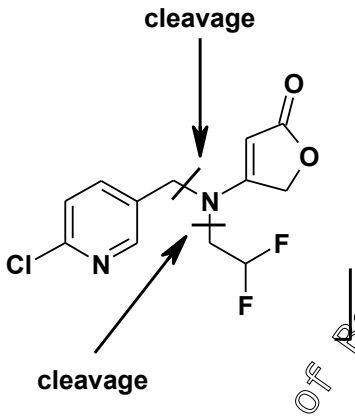
The principal metabolic reactions of [ethyl-1-^{14}C]BYI 02960 in rats were:

- Hydroxylation followed by conjugation with glucuronic acid,
- cleavage of the difluoroethyl group leading to BYI 02960-DFA and
- cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

Figure 5.1.3-05 schematically shows the positions in the molecule, which are involved in these metabolic reactions.

The proposed biotransformation pathway of [ethyl-1-^{14}C] BYI 02960 is presented in Figure 5.1.3-06.

Figure 5.1.3-05: Positions involved in metabolic degradation of BYI 02960



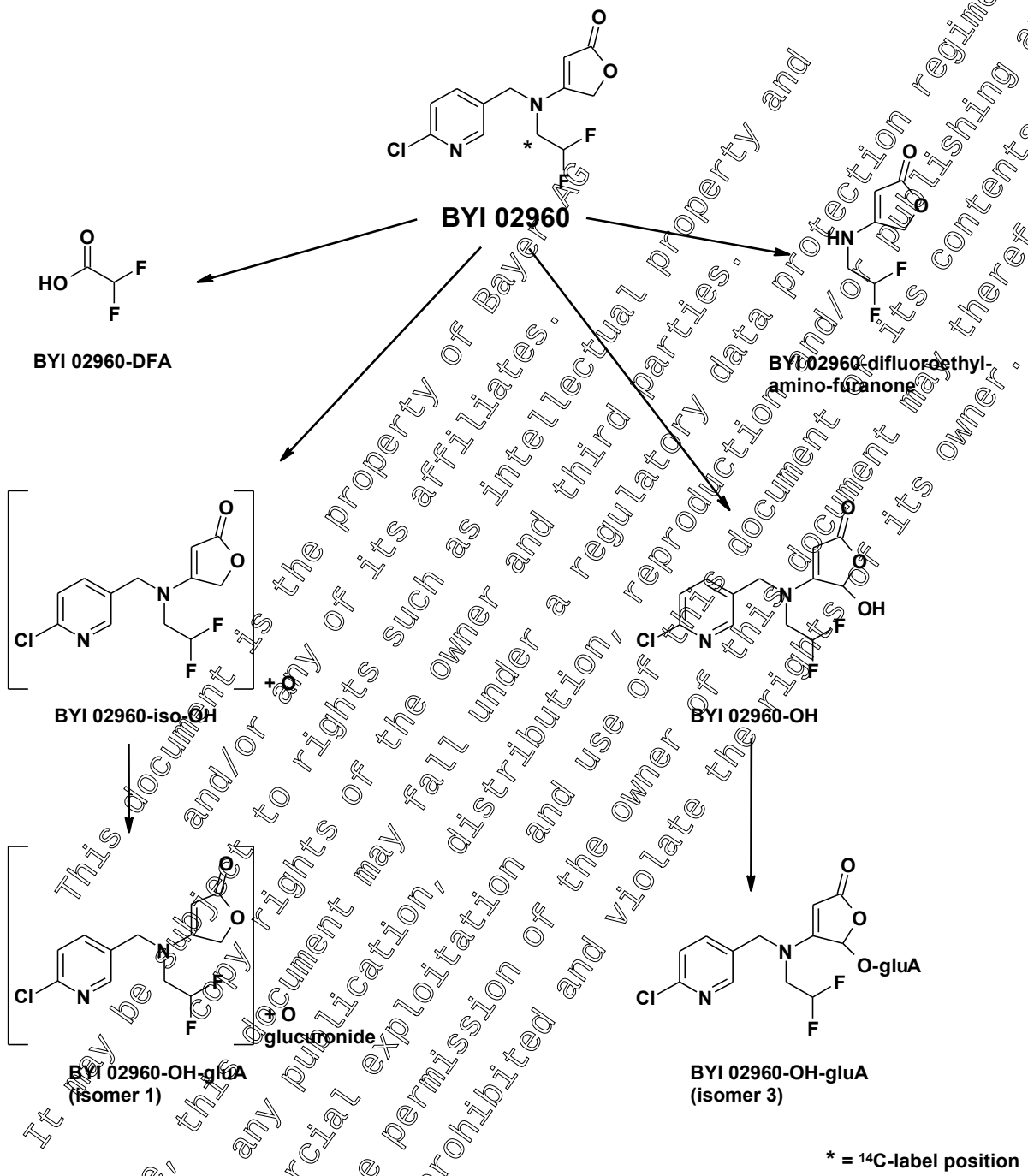
G. Conclusions

The kinetic and metabolic behavior of [ethyl-1-^{14}C]BYI 02960 in male and female Wistar rats can be characterized by the following observations:

- The distribution of the radioactivity within the organs and tissues (*i.e.* blood, liver, kidney, muscle and fat) was fast and showed a distinctive preference for liver and kidney as the main metabolizing and excretory organs.
- As anticipated, the highest radioactivity concentrations were measured in plasma, and in organs and tissues at one hour after administration. They decreased significantly within the test period of 24 hours. There were no indications of irreversible binding or retention of radioactivity in organs and tissues.
- Parent compound, major and several minor metabolites were identified in all samples. Identification rates were high with ≥ 95% of radioactivity in urine and approx. 93 - 100% of the radioactivity in plasma, organs and tissues.
- The metabolic transformation of BYI 02960 was principally oxidative in nature and took place at least at 3 different structural positions of the molecule.
- BYI 02960-DFA was by far the dominating metabolite in plasma, organs and tissues. On the other hand, the parent compound was the main constituent in urine. The metabolic pattern was in good accordance with that obtained from the corresponding ADME rat study (KIIA 5.1.3/01). With regard to the extent of metabolism, a clear sex difference was observed since it was higher in male than in female rats. The metabolic degradation of the parent compound was much less pronounced in females as compared to males.

Many examples of sex differences of metabolism in rats have been reported. A very important and general observation is the approximately threefold difference in the activity of hepatic microsomal monooxygenase (cytochrome P-450) in male as compared to female rats. These results are possibly due to the effects of hormones (sex, growth, and thyroid hormones) but also by other chemicals (references are provided in the report).

Figure 5.1.3-06: Proposed metabolic pathway of [ethyl-1-^{14}C]BYI 02960 in male and female rats based on metabolites detected in urine, plasma, liver, kidney, muscle and fat



KIIA 5.1.4 - Toxicokinetic studies - Repeated dose, oral route, in rats

All single dose experiments revealed no indication of a potential for retention, accumulation and/or persistence of the administered radioactivity in organs or tissues. This observation is supported by the low log P_{OW} of BYI 02960 of 1.2. Therefore, in line with para 26 of the OECD Test Guideline 417 (July 22nd, 2010), a repeated dose study was not considered necessary.

KIIA 5.2 - Acute toxicity

The potential for acute toxicity was evaluated for BYI 02960 technical, as well as the formulated products BYI 02960 200 SL and BYI 02960 480 FS. Acute toxicity results of the formulated products are summarized in Annex III. All studies were fully compliant with Good Laboratory Practice (GLP) and were conducted in accordance with prevailing OECD, EU, USEPA and Japanese MAFF testing guidelines.

KIIA 5.2.1 - Acute toxicity of BYI 02960 technical

The acute toxicity of BYI 02960 (96.2 % of purity) was low for all routes evaluated (oral, dermal and inhalational). The oral LD_{50} cut-off for rats was equal to 2 000 mg/kg body weight (bw) with mortalities reported at 2 000 mg/kg but none at 300 mg/kg. The rat acute dermal LD_{50} was > 2000 mg/kg bw. The rat acute inhalation LC_{50} (4-hour) was > 4671 mg/m^3, which was the highest achievable concentration. There were no mortalities, but there were transient clinical signs which were reversible within 3 days.

BYI 02960 was not irritating to rabbit skin and caused only slight ocular irritation (redness of the conjunctivae) which reversed within 48 hours. No evidence of skin sensitization (delayed contact hypersensitivity) was seen in a modified LLNA test (IMDS) in NMRI mice.

Table 5.2-01: Summary of acute toxicity data for BYI 02960 technical

Type of study (Document N°)	Species	Results	EPA Toxicity Category	GHS Classification (proposed)
Oral route M-349992-01-2	Rat	Mortalities observed at 2000 mg/kg; none at 300 mg/kg	II	Category 4 (LD_{50} cut off = 2000 mg/kg)
Dermal route M-349995-01-2	Rat	LD_{50} > 2 000 mg/kg	III	Category 5 / Unclassified
Inhalation M-362791-01-2	Rat	LC_{50} at 4 hours > 4671 mg/m^3	IV	Category 5 / Unclassified
Primary skin irritation M-353761-01-2	Rabbit	Non irritating	III	Category 5 / Unclassified
Eye irritation M-361319-02-2	Rabbit	Slight redness of the conjunctivae, reversed within 48 hours	IV	Category 5 / Unclassified
Skin sensitization M-353715-01-2	Mouse	Not sensitizing	Not Applicable	Category 5 / Unclassified

Report:	KIIA 5.2.1/01, [redacted] U.; 2009
Title:	BYI 02960, Acute toxicity in the rat after oral administration
Report No & Document No	AT05287 M-349992-01-2
Guidelines:	OECD 423 (2001); EEC Directive 440/2008 Part B – Method B.1.tris; EPA Health Effects test Guidelines (OPPTS 870.1100) (1998)
GLP	Yes (certified laboratory)

Executive Summary

In an acute oral toxicity study using a stepwise procedure, four groups of three fasted, young adult female Wistar rats (HsdCpb:Wu) were given successively a single oral dose of BYI 02960 (batch 2009-000239, 96.2% purity) in 2% Cremophor EL of 2000 mg/kg bw or 300 mg/kg bw and were observed for 14 days.

The dose of 2000 mg/kg bw induced mortalities (3/3 animals in the first group and 3/3 animals in the second group) and clinical signs such as decreased motility, tremor, piloerection, labored breathing and clonical cramps. No mortality was observed at 300 mg/kg, but breathing sounds were reported.

According to OECD guideline 423, the LD_{50} cut-off of BYI 02960 was 2000 mg/kg bw (GHS category 4, EPA toxicity category II).

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Beige powder
Lot/Batch: 2009-000239
Purity: 96.2%
CAS: 951659-40-8
Stability of test compound: Stable at 5 and 200 mg/mL at room temperature for 2 hours

2. Vehicle and/or positive control: 2% Cremophor EL in tap water

3. Test animals:
Species: Rat
Strain: HsdCpb:Wu
Age: 8 to 12 weeks approximately
Weight at dosing: 163 to 190 g
Source: [redacted] Netherlands
Acclimation period: At least 5 days
Diet: Provimi Kliba 3883 PM S15 Maus/Ratte Haltung, Kaiseraugst Switzerland, *ad libitum*
Water: Tap water, *ad libitum*

Housing:	Animals were group caged conventionally in polycarbonate cages on low dust wood granulate bedding	
Environmental conditions:	Temperature:	22 ± 2 °C
	Humidity:	55 ± 5%
	Air changes:	Approximately 10 changes per hour
	Photoperiod:	Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates
18 March to 22 April, 2009.

2. Animal assignment and treatment
The substance was tested using a stepwise procedure, each step using three female rats. The animals were assigned to their groups by randomization. The random list was based on evenly distributed chance numbers by a software application. Following an overnight fast (16 to 24 hours), two groups received a single dose of 2 000 mg/kg bw or 300 mg/kg bw of BYI 02960 (96.2% purity) by gavage. The test substance was administered in tap water with 2% Cremophor EL at a volume of 10 mL/kg bw. Clinical signs and mortality rates were determined several times on the day of administration and subsequently at least once daily for an observation period of at least 14 days. Body weights were recorded on days 1, 8 and 15. On day 15, surviving animals were sacrificed and all animals were necropsied and examined for gross pathological changes.

Table 5.2.1-01: Doses, mortality / animals treated

Dose (mg/kg bw)	Toxicological results*	Occurrence of signs	Time of death
2 000 (1st)	1/3/3	35 minutes - 3 days	2 hours
2 000 (2nd)	3/3/3	20 minutes - 3 hours	3 hours
300 (1st)	0/3/3	50 minutes - 7 hours	-
300 (2nd)	0/3/3	2 hours - 6 hours	-

* : number of animals which died spontaneously and/or were sacrificed in moribund state/number of animals with signs of toxicity/total number of animals used per group

3. Statistics
The data did not warrant statistical analysis.

II. Results and discussion

A. Mortality
Details are provided in table IIA 5.2.1-1. The dose of 2 000 mg/kg bw induced mortalities (1/3 animals in the first group and 3/3 animals in the second group), whereas no mortality was observed at 300 mg/kg/
The oral LD50 cut-off was 2 000 mg /kg bw according to OECD guideline 423.

B. Clinical observations

Clinical signs such as decreased motility, tremor, piloerection, labored breathing and clonical cramps were observed at 2000 mg/kg and breathing sounds were recorded at 300 mg/kg.

C. Body weight

There was no toxicological effect on body weight or body weight gain in rats treated at 300 mg/kg and in the surviving animals treated at 2000 mg/kg.

D. Necropsy

No abnormalities were observed at gross necropsy.

III. Conclusions

The oral LD_{50} cut off of BYI 02960 was 2000 mg/kg bw (GHS Category 4, EPA toxicity category II).

KIIA 5.2.2 - Acute percutaneous toxicity

Report:	**KIIA 5.2.1.2/01, [redacted] U.; 2009**
Title:	BYI 02960, Acute toxicity in the rat after dermal application
Report No & Document No	AT 05288 M-349995-01-2
Guidelines:	OECD 402 (1987); EEC Directive 440/2008 – Method B.3.;EPA Health Effects Test Guidelines (OPPTS 870.1200; 1998)
GLP	Yes (certified laboratory)

Executive Summary

In an acute dermal toxicity study, groups of young adult Wistar rats, 5/sex, were exposed by the dermal route to BYI 02960 (batch number 2009-000239, 96.2% purity). The test material was applied as received moistened with water to 10% of each animal's body surface at a dose of 2 000 mg/kg bw and left in contact with the skin for 24 hours. Animals were observed for the following 14 days.

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

BYI 02960 was regarded as non-toxic after dermal application. No clinical signs, no effect on body weight and no gross pathological findings were observed during the study. On the basis of this study, BYI 02960 does not warrant classification for dermal toxicity (EPA toxicity category III).

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Beige powder
Lot/Batch: 2009-000239
Purity: 96.2%
CAS: 951659-40-8

2. Vehicle and /or positive control: Test material dosed as received moistened with water

3. Test animals:
Species: Rat
Strain: HsdCpb:Wu
Age: 9 to 13 weeks approximately
Weight at dosing: 277 to 291 g for the males; 211 to 225 g for the females
Source: [redacted] Netherlands
Acclimation period: At least 5 days

Diet:	Provimi Kliba 3883 PM S15 Maus/Ratte Haltung, Kaiseraugst Switzerland, *ad libitum*	
Water:	Tap water, *ad libitum*	
Housing:	Animals were caged individually in polycarbonate cages on low dust wood granulate bedding	
Environmental conditions:	Temperature:	22 ± 2 °C
	Humidity:	55 ± 5%
	Air changes:	Approximately 10 changes per hour
	Photoperiod:	Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates
18 March to 01 April, 2009.

2. Animal assignment and treatment
Animals were assigned by randomization to the test groups listed in Table IIA 5.2.2.-1. The random list was based on evenly distributed chance numbers especially generated for the study by a software application. On the day prior to dosing, the fur was clipped from the dorsal area of the trunk of each animal (approximately 10% of the body surface area). The test substance was administered as a single occluded dermal application and was applied moistened with distilled water. After an exposure period of 24 hours, the occlusion was removed and residual test material was removed with tepid water using soap and gently patting the area dry. Animals were observed for clinical signs and mortality several times on the day of dosing and subsequently at least once daily for an observation period of at least 14 days. Individual body weights were recorded on days 1, 8 and 15. On day 15, all animals were sacrificed by carbon dioxide and were necropsied and examined for gross pathological changes.

Table 5.2.2-01: Doses, toxicological results*/ animals treated

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

*** : number of animals which died spontaneously and/or were sacrificed in moribund state/number of animals with signs of toxicity/total number of animals used per group**

3. Statistics
The data did not warrant statistical analysis.

II. Results and discussion

A. Mortality

Details are provided in table IIA 5.2.2.-1. No mortalities occurred at 2000 mg/kg bw, the only dose level tested.

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

B. Clinical observations

No clinical signs were observed during the study.

C. Body weight

Body weight and body weight gain of male or female rats were not affected by treatment.

D. Necropsy

The necropsies performed at the end of the study revealed no particular findings.

III. Conclusions

The dermal LD_{50} of BYI 02960 was higher than 2000 mg/kg bw in both sexes (GHS category 5, unclassified; EPA toxicity category III).

KIIA 5.2.3 - Acute inhalation toxicity

Report:	**KIIA 5.2.3/01, [redacted] A.; 2010**
Title:	BYI 02960, Acute inhalation toxicity in rats
Report No & Document No	AT05727 M-362791-01-2
Guidelines:	OECD 403 (1981); EEC Directive 92/69 Annex V - Method B.2. (1992); EPA Health Effects Test Guidelines (OPPTS 870.1300; 1998); Japan MAFF, Notification N° 12 Nousan-8147 (2000)
GLP	Yes (certified laboratory)

Executive Summary

In an acute inhalation study, groups of young adult Wistar rats (5/sex) were exposed by the inhalation route to BYI 02960 (batch number 2009-000239, 96.2% purity) in air for 4 hours (nose only) at a concentration of 4671 mg/m^3. A concurrent control group was exposed to an atmosphere using similar exposure conditions (15 L/min conditioned dry air). Animals were observed for the following 14 days.

The inhalation LC_{50} for the males was > 4671 mg/m^3
for the females was > 4671 mg/m^3
for the combined sexes was > 4671 mg/m^3

BYI 02960 (liquid aerosol) proved to be essentially acutely non-toxic via the inhalation route to rats. No mortality occurred up to the maximum technically attainable concentration. The rats displayed the following transient clinical signs: increased breathing rate or in labored or irregular patterns, piloerection, motility reduced or increased, anxiety, tremor, limp, gait high-legged, exophthalmia, nose red encrustations, stridor, abdominal position with uncoordinated movements and hypothermia. 48 hours after exposure all rats were without clinical signs. No treatment-related significant effects were noted on body weight evolution in males. A very slight decrease in female body weight was observed on day 1. At necropsy no treatment-related findings were reported. On the basis of this study, BYI 02960 does not warrant classification as being harmful or toxic (EPA toxicity category IV).

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: beige powder
Lot/Batch: 2009-000239
Purity: 96.2%
CAS: 951659-40-8
Stability of test compound: stable at 70 °C for at least 24 hours

2. Vehicle and /or positive control: The test substance was aerosolized as 50% (w/w) solution in PEG 400 (Lutrol)

3. Test animals:

Species:	Rat
Strain:	HsdCpb:Wu (SPF)
Age:	2 to 3 months approximately
Weight at dosing:	160 to 183 g for the males and 167 to 184 g for the females
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Provimi Kliba 3883 = NAFAG 9441 pellets maintenance diet for rats and mice, Kaiseraugst Switzerland, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were individually caged in conventional Makrolon® Type III_H cages
Environmental conditions:	Temperature: 22 ± 3 °C Humidity: 50 ± 10% Air changes: Approximately 10 changes per hour Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates
31 March to 21 April, 2009.

2. Animal assignment and treatment
Animals were assigned to the test groups listed in Table IIA 5.2.3-1. The computerized list of random numbers served the purpose to assign the animals at random to the treatment groups. Animals were exposed to the aerosolized test substance in Plexiglas exposure tubes applying a directed-flow nose-only exposure principle. Animals were examined carefully several times on the day of exposure and at least once daily thereafter for 2 weeks. The following reflexes were tested: visual placing response, grip strength on wire mesh, abdominal muscle tone, corneal and pupillary reflexes, pinnal reflex, righting reflex, tail-pinch response, startle reflex with respect to behavioral changes stimulated by sounds (finger snapping) and touch (back). The rectal temperatures were measured shortly after cessation of exposure. Individual body weights were recorded before exposure and on days 3, 7 and 14. On day 15, all animals were sacrificed, necropsied and examined for gross pathological changes.

Table 5.2.3-01: Doses, mortality / animals treated

Analytical concentration (mg/m³)	Male	Female	Combined
0	0/5	0/5	0/10
467	0/5	0/5	0/10

3. Generation of the test atmosphere / chamber description
Directed-flow nose-only inhalation chambers (TSE, 61348 Bad Homburg) were used. During pre-study optimization, it was demonstrated that the limit concentration (target concentration 5000 mg/m3) was attainable using a test item solution of 50% (w/w) in PEG 400 (Lutrol). The test substance concentration was determined by gravimetric analysis. Chamber samples were collected after the

equilibrium concentration had been attained in hourly intervals. Two samples during each exposure were also taken for the analysis of the particle-size distribution using an Andersen cascade impactor.

Table 5.2.3-02: Concentrations

Groups	1	2
Target Concentration (mg/m³)	0	5000
Gravimetric concentration (mg/m³)	5135[a]	10867.5[b]
Actual Concentration (mg/m³)	-	5434
Analytical concentration (mg/m³)	-	4671
Mass median aerodynamic diameter (µm)	1.87	2.04
Geometric standard deviation	2.09	1.86
Aerosol Mass < 3 µm (%)	74	73.3

[a]: Lutrol (PEG 400); [b]: 50% BYI 02960 solution in Lutrol (PEG 400)

4. Statistics

A one-way ANOVA (vide infra) was used to analyze body weight gain data and rectal temperature measurements. Specific findings from the respiratory tract were evaluated statistically using the pair-wise Fisher test after the R x C chi-squared test. The Fisher test was only performed if differences occurred between groups in the R x C chi-squared test or if a frequency value of < 5 was calculated. This procedure was performed in accordance with Gad and Weil (1982). For calculation of the unilateral p value a symmetrical distribution was assumed (p unilateral = (p bilateral)/2).

II. Results and discussion

A. Mortality

No mortality occurred in any group of animals exposed.

The 4 hour inhalation LC_{50} for the males was > 4671 mg/m³
for the females was > 4671 mg/m³
for the combined sexes was > 4671 mg/m³

B. Clinical observations

The rats from the treated group (group 2) displayed the following transient clinical signs: increased breathing rate or in labored or irregular patterns, piloerection, motility reduced or increased, anxiety, tremor, limp, gait high-legged, exophthalmia, nose red encrustations, stridor, abdominal position with uncoordinated movements. 48 hours after exposure, all rats were without clinical signs. A battery of reflex measurements was recorded on the first post exposure day. Measurements from the rats revealed normal reflexes. A significant decrease in body temperature was observed in the treated animals.

Table 5.2.3-03: Clinical signs and rectal temperature

Groups/sex	Target concentration mg/m³	Toxicological results [a]	Onset and duration of signs	Rectal temperature
1/males	0	0/0/5	-	37.9
2/males	5000	0/5/5	0 d - 1 d	33.6**
1/females	0	0/0/5	-	38.2
2/females	5000	0/5/5	0 d - 1 d	33.4**

[a]: number of dead animals/number of animals with signs after cessation of exposure/number of animals exposed
** : $p < 0.01$

C. Body weight

No effects on body weight were observed in the males. A slight non statistically significant body weight decrease (approximately - 3%) was observed in the treated females one day after exposure.

D. Necropsy

Macroscopic changes causally related to the exposure of the test article were not observed.

III. Conclusions

The acute inhalation LC_{50} of BYI 02960 for the combined sexes was 4671 mg/m³. BYI 02960 does not warrant classification as being toxic or harmful on the basis of its acute inhalation toxicity (EPA toxicity category IV).

KIIA 5.2.4 – Skin irritation

Report:	**KIIA 5.2.4/01, [redacted] C.; 2009**
Title:	BYI 02960, Acute skin irritation/corrosion on rabbits
Report No & Document No	AT05342 M-353761-01-2
Guidelines:	OECD 404 (2002); EEC Directive 440/2008; EPA Health Effects Test Guideline (OPPTS 870.2500; 1998)
GLP	Yes (certified laboratory)

Executive Summary

In a primary dermal irritation study, 3 young adult New Zealand female rabbits were exposed via the dermal route to 0.5 g of pulverized test substance which was moistened with water (Batch number 2009-000239, 96.2% purity) per animal. In the first step only one animal was used and three patches were applied successively to this animal. The first patch was removed after three minutes. As no serious skin reactions were observed, the second patch was removed after one hour and then the third patch applied and removed after four hours. The test was completed using two additional animals exposed for four hours. The animals were observed for 72 hours.

No erythema, eschar or œdema were observed at any time point. In this study, BYI 02960 was not a dermal irritant and does not warrant classification as being irritating to the skin (EPA toxicity category IV).

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Beige powder
Lot/Batch: 2009-000239
Purity: 96.2%
CAS: 951659-40-8
Stability of test compound: Technical material specified for the duration of the study

2. Vehicle and /or positive control: Test material dosed as received moistened with water

3. Test animals:
Species: Rabbit
Strain: Crl:KBL(NZW)BR
Age: Young adult females
Weight at dosing: 2.8 to 3.5 kg
Source: [redacted] Germany
Acclimation period: At least 5 days
Diet: [redacted] K-Z 4mm ([redacted], Germany), approximately 100 g per animal per day
Water: Tap water, *ad libitum*

Housing:	Animals were caged individually in cage units Metall/Noryl by EBECO	
Environmental conditions:	Temperature:	20 ± 3 °C
	Humidity:	50 ± 25%
	Air changes:	Not mentioned
	Photoperiod:	Alternating 12-hour light and dark cycles.

B. Study Design and methods

1. In life dates
31 March to 03 April, 2009.

2. Animal assignment and treatment
On the day prior to dosing, the fur was clipped on the right and left side from the dorsal-lateral area of the trunk of each rabbit. Care was taken to avoid abrading the skin. 0.5 g of the pulverized test substance moistened with water was applied to the skin of the animals under a gauze patch. The treated area was approximately 2.5 cm by 2.5 cm in size. The patch was placed on the dorso-lateral areas of the trunk of each rabbit and was held in place with non-irritating tape for the duration of the exposure period. After the exposure period the dressing and the patch were removed and the exposed skin area was carefully washed with water without altering the existing response, or the integrity of the epidermis. The surrounding untreated skin served as control.

In the first step only one animal was used and three patches were applied successively to this animal. The first patch was removed after three minutes. As no serious skin reactions were observed, the second patch was removed after one hour and then the third patch applied and removed after four hours. The test was completed using two additional animals exposed for four hours. The responses were graded one hour later.

The dermal irritation was scored at 1, 24, 48 and 72 hours after patch removal. If no irritation indices were observed after 72 hours, the study was finished. If dermal irritation was observed, animals were monitored usually on days 7 and 14 after patch removal. The degree of erythema/eschar formation and oedema formation was recorded as specified by Draize, and any serious lesion or toxic effects other than dermal irritation were also recorded. The body weight of each animal was recorded at the beginning of the study.

II. Results and discussion

A. Findings

No erythema, eschar or oedema was observed at any time point.

Table 5.2.4-01: Individual skin irritation scores according to the Draize scheme on the first animal

Observation (immediately after patch removal)	Duration of exposure	
	3 minutes	1 hour
Erythema (redness) And eschar formation	0	0
Oedema formation	0	0

Table 5.2.4-02: Individual and mean skin irritation scores after 4 hour exposure according the Draize scheme

	Erythema and eschar			Oedema		
Animal number (body weight in kg)	**1 (2.8)**	**2 (3.5)**	**3 (3.4)**	**1 (2.8)**	**2 (3.5)**	**3 (3.4)**
1 hour	0	0	0	0	0	0
24 hours	0	0	0	0	0	0
48 hours	0	0	0	0	0	0
72 hours	0	0	0	0	0	0
Mean score 24-72 hours	0			0		
No positive response: mean scores < 2 = - Positive response : mean scores ≥ 2 = +						

III. Conclusions

BYI 02960 was non-irritant to the rabbit skin and there were no systemic intolerance reactions. On the basis of this study, BYI 02960 does not warrant classification as being irritating to the skin (EPA toxicity category IV).

KIIA 5.2.5 - Eye irritation

Report:	KIIA 5.2.5/01, [redacted] C.; 2009
Title:	BYI 02960, Acute eye irritation on rabbits, amended report
Report No & Document No	AT05341 M-361319-02-2
Guidelines:	OECD 405 (2002); EEC Directive 440/2008; EPA Health Effects Test Guideline (OPPTS 870.2400; 1998)
GLP	Yes (certified laboratory)

Executive Summary

In a primary eye irritation study, 0.1 g of pulverized test substance (Batch number 2009-000239, 96.2% purity) was placed into the conjunctival sac of one eye of a rabbit after having gently pulled the lower lid away from the eyeball. The other eye, which remained untreated, served as control. Since severe irritation was not observed one hour after treatment, two further rabbits were treated as described. Eye irritation was scored and recorded at 1, 24, 48 and 72 hours after application. As no irritation indices were observed after 72 hours, the study was finished.
The degree of ocular lesions was recorded as specified by Draize and any serious lesion or toxic effects other than ocular lesions were also recorded. Body weight of each animal was recorded at the beginning of the study.
A slight redness of the conjunctivae was observed after 1 and 24 hours in all females (grade 2 for 3/3 females after 1 hour, grade 1 for 2/3 females and grade 2 in 1/3 females after 24 hours). According to the classification criteria, BYI 02960 was slightly irritating to the eye and there were no systemic intolerance reactions (EPA toxicity category III).

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Beige powder
Lot/Batch: 2009-000239
Purity: 96.2%
CAS: 951659-40-8
Stability of test compound: Technical material specified for the duration of the study

2. Vehicle and /or positive control: Test material dosed as received

3. Test animals:
Species: Rabbit
Strain: Crl:KBL(NZW)BR
Age: Young adult females
Weight at dosing: 3.2 to 3.5 kg
Source: [redacted] Germany
Acclimation period: At least 5 days

Diet:	[redacted]K-Z 4mm [redacted], Germany), approximately 100 g per animal per day	
Water:	Tap water, *ad libitum*	
Housing:	Animals were caged individually in cage units Metall/Nord by EBECO	
Environmental conditions:	Temperature:	20 ± 3 °C
	Humidity:	50 ± 25%
	Air changes:	Not mentioned
	Photoperiod:	Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates
08 to 11 April, 2009.

2. Animal assignment and treatment
The testing strategy comprised a stepwise approach including the evaluation of existing data, the performance of a SAR evaluation for eye and skin corrosion/irritation, measurement of pH value, the evaluation of data on systemic toxicity via the dermal route, the performance of a validated *in vitro* test for skin corrosion (Human 3D Epidermal Skin Model) and *in vivo* testing for skin irritation/corrosion in rabbits before *in vivo* testing for eye irritation/corrosion in rabbits. The test compound is not corrosive to the skin.

On the day before dosing, both eyes of each animal were examined including fluorescein examination. Only animals with healthy intact eyes were used. 0.1 g of pulverized test substance was placed into the conjunctival sac of one eye of the first animal after having gently pulled the lower lid away from the eyeball. The lids were gently held together for about one second in order. to prevent loss of the test substance. The other eye, which remained untreated, served as control. The eye was not rinsed for at least 24 hours following installation. One hour after treatment a severe irritation was not observed, so two further rabbits were treated as described. The eye irritation was scored and recorded at 1, 24, 48 and 72 hours after application. If no irritation was observed after 72 hours, the study was finished. If eye irritation was observed, animals were monitored usually on days 7, 14 and 21 after application until the changes had completely subsided, however for not more than 21 days after application.
The degree of ocular lesions was recorded as specified by Draize, and any serious lesion or toxic effects other than ocular lesions were also recorded. Body weight of each animal was recorded at the beginning of the study.

II. Results

Redness of the conjunctivae was observed after 1 and 24 hours in all females (grade 2 for 3/3 females after 1 hour, grade 1 for 2/3 females and grade 2 in 1/3 females after 24 hours).

Table 5.2.5-01: Eye irritation scores according to the Draize scheme

	Cornea			Iris			Conjunctiva-redness			Conjunctiva-chemosis		
Animal number	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Time of observation												
1 hour	0	0	0	0	0	0	2	2	2	0	0	1
24 hours	0	0	0	0	0	0	2	1	1	1	0	0
48 hours	0	0	0	0	0	0	0	0	0	0	0	0
72 hours	0	0	0	0	0	0	0	0	0	0	0	0
Mean scores 24-72 hours	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.7**	**0.3**	**0.3**	**0.3**	**0.0**	**0.0**

Conjunctivae: Redness (refers to palpebral and bulbar conjunctivae; excluding cornea and iris)
0: Normal
1: Some blood vessels hyperaemic (injected)
2: Diffuse, crimson color; individual vessels not easily discernible

Chemosis: Swelling (refers to lids and/or nictating membranes)
0: Normal
1: Some swelling above normal
2: Obvious swelling with partial eversion of lids
3: Swelling, with lids about half closed
4: Swelling, with lids more than half closed

III. Conclusions

Slight ocular irritation was observed in all animals but had reversed by 48 hours. On the basis of this study, BYI 02960 does not warrant classification as being an eye irritant in the EU and qualifies for EPA toxicity category III.

KIIA 5.2.6 - Skin sensitization

Report:	**KIIA 5.2.6/01, [redacted] H.-W.; 2009**
Title:	BYI 02960, Local lymph node assay in mice (LLNA/IMDS)
Report No & Document No	AT05334 M-353715-01-2
Guidelines:	OECD 406 (1992) and 429 (2002); EEC Directive 2004/73/EC Annex V – Method B.6, (1996) and B42 (2001); EPA Health Effects Test Guideline (OPPTS 870.2600; 2003)
GLP	Yes (certified laboratory)

Executive Summary

The modified Local Lymph Node Assay (IMDS) was performed on 24 female NMRI mice (6 animals/test item group and 6 control animals) to determine if there was any specific (sensitizing) or non-specific (irritant) stimulating potential of the test item BYI 02960. The dosing formulations at 0, 2%, 10% and 50% were prepared by dissolving the test substance in DMF.
Compared to vehicle-treated animals, none of the parameters measured in the substance-treated groups (cell counts and weights of draining lymph nodes, ear weights and ear swelling) reached or exceeded the positive levels defined for this assay.
BYI 02960 has no sensitizing potential in mice after dermal application of up to and including a 50% concentration.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Beige powder
Lot/Batch: 2009-000239
Purity: 96.2%
CAS: 951659-40-8
Stability of test compound: Stable in vehicle at 1 and 50 % for at least 4 days at room temperature

2. Vehicle and /or positive control: Test material formulated in DMF

3. Test animals:
Species: Mice
Strain: Hsd Win: NMRI
Age: 8 weeks old females
Weight at dosing: 26 to 32 g
Source: [redacted] Nederland
Acclimation period: At least 7 days
Diet: Provimi Kliba SA 3883 - maintenance Diet for rats and mice - (supplied by Provimi Kliba SA, CH-4303 Kaiseraugst), *ad libitum*
Water: Tap water, *ad libitum*

Housing: Animals were caged by up to 8 in type III Makrolon® cages during acclimation. Single-housed in type II cages during the study

Environmental conditions:
Temperature: 22 ± 2 °C
Humidity: 55 ± 15%
Air changes: At least 10 times per hour
Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates
17 to 20 March, 2009.

2. Animal assignment and treatment
The sensitivity and reliability of the method were checked in a previous study where female NMRI mice were administered with Alpha Hexyl Cinnamic Aldehyde formulated in different vehicles (PEG 400, DAE 433, DMF, MEK, acetone/olive oil (4:1) and cremophor EL/physiological saline solution 2%v/v) at concentrations of 3%, 10% and 30%.
During this study, six animals were placed in each group. They received the test item formulated once on day 1 of the study in DMF at 0%, 2%, 10% or 50% through epicutaneous application onto the dorsal part of both ears of the animals. This treatment was repeated on three consecutive days. The volume administered was 25 µl/ear.

Table 5.2.6-01: Experimental study design: Concentrations and groups

Group	Test Substance(s)	Concentration(s) (%) Days 1 - 3	Number of animals per group
1	DMF	0	6
2	BYI 02960 in DMF	2	6
3		10	6
4		50	6

On Day 4, animals were anaesthetized by inhalation of carbon dioxide and sacrificed one day after the last application. The appropriate organs were then removed. Lymphatic organs (auricular lymph nodes) were transferred into physiological saline (PBS).

3. Lymph node weight and cell count determination
The weight of the lymph nodes was determined and, after crushing the lymph nodes through a sieve into a 12-well plate, the cell counts per mL were determined using a multisizer from Coulter Electronics.
The stimulation index is calculated by dividing the absolute number of weight or cells counts of the substance treated lymph nodes by the vehicle treated ones. Thus in case of no stimulating effect the index is always around 1 (± standard deviation) and the indices of vehicle treated animals are set at 1 (± standard deviation).

4. Ear swelling

Before the first treatment and before sacrifice the thickness of both auricles of the animals was measured using a spring-loaded micrometer.

5. Ear weight

On day 4 of the study the ear weight of sacrificed animals was measured using a punch to take an 8 mm diameter piece of every ear.

6. Body weight

The body weights of the animals were recorded at the start and the end of the study (day 1 and day 4).

7. Statistical analyses

When it was statistically reasonable, the values from treated groups were compared with those from the control group(vehicle) by a one-way analysis of variance (ANOVA) when the variances were considered homogeneous according to a homogeneity testing like Cochran' s test. Alternatively, if the variances were considered to be heterogeneous ($p < 0.05$), a nonparametric Kruskal-Wallis test has been used (Kruskal-Wallis ANOVA) at significance levels of 5%. Two sided multiple test procedures were done according to Dunnett or Bonferroni-Holm, respectively. Outlying values in the LN weights were eliminated at a probability level of 99% by Nalimov's method.
In addition, for the LLNA/IMDS the smallest significant differences in the means were calculated by Scheffe's method, which according to Sachs can be used for both equal and unequal sample sizes.

II. Results and discussion

A. Stimulation Index

The NMRI mice did not show an increase in the stimulation indices for cell counts or for weights of the draining lymph nodes after application of the test item BYI 02960.
The "positive level" which is 1.4 for the cell count index was never reached or exceeded in any dose group.

Table 5.2.6-02: Summary table for lymph node weight and cell count indices

Doses (%)	Weight index		Cell count index	
	Mean	S.D. %	Mean	S.D. %
0	1.00	8.00	1.00	18.62
2	1.01	14.35	1.21	19.32
10	0.92	17.31	0.97	23.04
50	0.93	21.41	0.87	26.94

The "positive level" of ear swelling, which is 2 x l0-2 mm increase , *i.e.* about 10% of the control values, has not been reached or exceeded in any dose group

Table 5.2.6-03: Summary table for ear swelling (in 0.01 mm)

Doses (%)	Day 1		Day 4		Index Day 4
	Mean	S.D. %	Mean	S.D. %	
0	18.17	4.60	19.08	5.68	1.00
2	18.42	5.41	19.00	4.49	1.00
10	18.75	4.62	19.33	5.55	1.01
50	18.42	2.80	19.08	5.22	1.00

No substance specific effects were determined for ear weights either.

Table 5.2.6-04: Summary table for ear weight - in mg per 8 mm diameter punch

Doses (%)	Day 4		Index Day 4
	Mean	S.D. %	
0	12.88	7.31	1.00
2	12.93	6.68	1.00
10	12.86	4.81	1.00
50	12.54	7.86	0.97

B. Body weight

The body weights of the animals were not affected by any treatment.

III. Conclusions

This study points neither to a non-specific (irritant) nor to a specific irnmunostimulating (sensitizing) potential of the test item.

This applies to NMRI mice, for weight and cell counts of the draining lymph nodes as well as ear swelling and ear weight indices evaluated after application of BYI 02960.

Taken together, no antigen specific activation of the cells of the immune system via dermal route was determined after application of up to and including 50% BYI 02960 by the method used. Therefore, the concentration of 50% turned out to be the NOAEL for the parameters investigated in this study with respect to skin sensitization.

KIIA 5.2.7 - Potentiation/interactions of multiple active substances or products

Potentiation/interaction of multiple active ingredients is currently not a requirement in the EU, the USA or Canada. Hence, no such studies were conducted and data/documents do not need to be submitted.

KIIA 5.3 - Short-term toxicity

The short-term toxicity studies with BYI 02960 were conducted between 2007 and 2011. Several range finding studies, which were not fully compliant to GLP, were performed in early phases. All compulsory studies were in accordance with OECD, EU, USEPA and Japanese MAFF testing guidelines and were fully compliant with GLP. A summary of all these results is presented in Table 5.3-1.

Two 28-day rat studies were performed, one using a gavage administration and the other an administration through the diet. In the first gavage study, Wistar rats (5/sex/group) were administered at 75, 200 and 350 mg/kg/day. The vehicle used was corn oil supplemented with 10% ethanol and 10% water, v/v. Two females died on day 6 at 350 mg/kg/day. Not statistically significant lower mean body weight was observed at 350 mg/kg/day in the males throughout the study and in females during the first week. Lower total bilirubin and glucose concentrations were observed in both sexes at 350 mg/kg/day and in males only at 200 mg/kg/day. Higher triglyceride concentration was observed in both sexes at 350 mg/kg/day and in females only at 200 mg/kg/day. An increase in creatinine concentration and alanine aminotransferase and/or alkaline phosphatase activities were noted in females at 350 and 200 mg/kg/day. The target organs were the liver and the thyroid with higher absolute and/or relative liver weights and centrilobular hepatocellular hypertrophy observed in both sexes at 350 and 200 mg/kg/day and diffuse follicular cell hypertrophy observed in the thyroid in both sexes at 350 mg/kg/day and in males only at 200 mg/kg/day. BYI 02960 showed a cytochrome P450 3A family inducer profile with an increase in BROD activity observed in both sexes at 350 and 200 mg/kg/day. The NOAEL of this study was 75 mg/kg/day for both sexes.
As there were no significant differences between males and females in the first study, only male Wistar rats (5/group) were used in the second study. BYI 02960 was administered through the diet at 500 (actual analyzed concentration of 410 ppm equivalent to 33.6 mg/kg body weight/day) and 5000 ppm corresponding to 385 mg/kg body weight/day. Treatment-related findings were only observed at 5000 ppm. Lower mean body weight and food consumption were observed throughout the study period. Lower total bilirubin and glucose concentrations and higher urea and total cholesterol concentrations were observed at the end of the study. Hormone analysis showed an increase in TSH and a slight decrease in T4. The target organs were both the liver (with increased relative weight, prominent lobulation and centrilobular hepatocellular hypertrophy) and the thyroid gland (with diffuse follicular cell hypertrophy). Both BROD and UDPGT activities were increased at the end of the study. The NOEL in this study was 410 ppm equating to 33.6 mg/kg/day.
Wistar rats (10/sex/group) were administered at 100, 500 and 2500 ppm (equating to 6.0, 30.2 and 156 mg/kg/day in males and 7.6, 38.3 and 186 mg/kg/day in females) for at least 90 days. An additional 10 animals per sex were fed control or high dose test diet for at least 90 days and subsequently fed control diet and observed for reversibility or persistence of toxic effects after a post-treatment recovery period of at least 28 days. Significant findings were limited to the group treated at 2500 ppm, except for a reduced mean body weight gain observed in females during the first and the last weeks of the study. At 2500 ppm, a lower body weight was observed in both sexes throughout the study. Throughout the recovery phase of the study the mean body weight of males and females remained lower than the control group. A slight reduction in mean food consumption was observed for males during the first four days of the study and thereafter on several occasions and for the females from the first week of the study until Study Week 7. A higher mean platelet count was observed in females. Mean total bilirubin

and glucose concentrations were slightly lower in both sexes and mean total cholesterol and triglyceride concentrations were slightly higher when compared to the controls. The change observed for total bilirubin was considered to be partially reversible in females. The other treatment-related changes were considered to be reversible. The target organs were also the liver (with higher relative weights to body weight ratio and centrilobular hepatocellular hypertrophy in both sexes) and the thyroid gland (dark aspect at necropsy and minimal follicular cell hypertrophy in some males). These findings were totally reversible. The NOAEL in this study was 500 ppm equating to 30.2 and 38.3 mg/kg/day in males and females, respectively.

In a 28-day mouse study, BYI 02960 was administered to C57BL/6J mice at 300, 600 and 1200 ppm (equating to 50, 98 and 207 mg/kg/day in males and 59, 122 and 240 mg/kg/day in females). The only effect observed in this study was a slightly lower mean body weight in males on study day 8 at 1200 ppm. The NOAEL was considered to be 1200 ppm. Due to instability of BYI 02960 in rodent diet the actual concentration is considered to be in the region of 960 to 1080 ppm (166 to 186 mg/kg body weight/day for the males and 192 to 216 mg/kg body weight/day for the females, respectively).
In the 90-day mouse study, BYI 02960 was administered to C57BL/6J mice at 100, 500 and 2500 ppm (equating to 16, 81 and 407 mg/kg/day in males and 19, 98 and 473 mg/kg/day in females). Effects were limited to 2500 ppm except that lower mean body weight gain was observed in males during the first week of the study. A lower body weight was observed in both sexes throughout the study. A slight reduction in mean food consumption was observed in females between Study Days 1 and 22. A lower mean total cholesterol concentration, higher mean urea concentrations and slightly lower total protein concentrations were observed in both sexes, whilst higher mean alkaline phosphatase activity was noted in males and mean alanine and aspartate aminotransferase activities were higher in females. In females, mean albumin concentrations were slightly lower. The target organs were the liver (with higher mean absolute and relative weights in females, pale liver in the females and slight increase in severity of diffuse hepatocellular vacuolation in both sexes) and the kidney (with lower mean absolute and relative weights to brain weight ratio in males and loss of the normal multifocal/diffuse cortical epithelial vacuolation in males also). The NOAEL was 500 ppm (equating to 80.6 mg/kg body weight/day) in males and the NOAEL in females (equating to 98.1 mg/kg body weight/day).

Groups of two males and two females Beagle dogs received BYI 02960 mixed in their diet at concentrations of 0, 500, 2000 or 4000 ppm (equating approximately to 0, 16, 62, 118 mg/kg body weight/day in males and 0, 18, 77, 131 mg/kg body weight/day in females) for at least 28 days. At 4000 ppm, there was an overall body weight loss observed in one male whilst the body weight of the other male remained static. One female had an overall body weight gain of 0.7 kg whilst the body weight of the other female remained static. Lower food consumption was observed in both male and female animals. Hematology assessment revealed a slightly increased platelet count in both females and in 1/2 males at 4000 ppm and in 1/2 females at 2000 ppm. In isolation this treatment-related change was not considered to be adverse. The target organ was the liver with decreased centrilobular glycogen accumulation in incidence and/or severity in both sexes at 4000 ppm and in males only at 2000 ppm. This was considered to be a treatment-related but not adverse effect. The NOAEL in this study was 2000 ppm (equating to 62 and 77 mg/kg/day in males and females, respectively).
BYI 02960 was administered via the diet to beagle dogs (4/sex/dose) of both sexes at nominal concentrations of 0, 400, 1200 or 3600/2400 ppm for at least 90 days (equating approximately to 12, 33 or 102/85 mg/kg body weight/day in males and 12, 41 or 107/78 mg/kg body weight/day in females).

The 3600 ppm dose group was reduced to 2400 ppm beginning Study Week 9 due to clinical signs seen in two of the dogs on Day 44 and continual weight loss in the high-dose group.
In the high dose group, compound-related clinical findings were unsteady and stiff back legs and lower back on study days 44, 53, and 54 in one male and on study day 44 for one female. Lower body weight was observed in males and females, during the first week of the study at 3600/2400 ppm and in males only at 1200 ppm. Food consumption was also reduced at the beginning of the study in both sexes at 3600/2400 ppm and in males only at 1200 ppm. Higher creatine phosphokinase, aspartate aminotransferase, and alanine aminotransferase activities were observed at the 2-month test interval in both sexes at 3600/2400 and 1200 ppm. Lower red blood cell count, hemoglobin concentration, and hematocrit were observed at 3600/2400 ppm at 1, 2, and 3 months in both sexes. The target organs were the liver at 3600./2400 ppm (with higher absolute and relative weights in both males and females and minimal brown pigments in Kupffer cells in females), the kidney (with higher relative weights in both sexes at 3600/2400 ppm and males only at 1200 ppm) and skeletal muscle (with minimal to slight myofiber atrophy/degeneration in both sexes at 3600/2400 ppm and 1200 ppm). The NOEL in this study was 400 ppm for males and females equating to 12 mg/kg/day.

Male and female Beagle dogs (4/sex/dietary level) were fed control feed or feed containing BYI 02960 at dietary concentrations of 150, 300, or 1000 ppm (approximately equal to 4.6/4.1, 7.8/7.8, 28.1/28.2 mg/kg body weight/day in males/females, respectively) for at least one year. Test substance-related effects were limited to degeneration noted in skeletal muscle (gastrocnemius, biceps femoris) of males and females at 1000 ppm only. Minimal to slight, focal to multifocal areas of degeneration of skeletal muscles were noted in males (gastrocnemius - incidence 2/4, biceps femoris - incidence 3/4) and females (gastrocnemius - incidence 2/4, biceps femoris - incidence 3/4). Degeneration of the myofiber comprised one or more of the following changes: atrophy, necrosis, and/or presence of inflammatory cells around the affected myofiber. Skeletal muscles evaluated from 150 ppm and 300 ppm males and females were comparable to controls. Based on the micropathology findings, the lowest-observed-adverse-effect-level (LOAEL) in this study was 1000 ppm, which was equivalent to 28.1 and 28.2 mg/kg body weight/day for male and female dogs, respectively. Based on the lack of adverse compound-related effects, a dietary level of 300 ppm (equivalent to 7.8 mg/kg body weight/day for both sexes) was considered to be a no-observed-adverse-effect-level (NOAEL).

Male and female Wistar rats (10/sex/group) were administered vehicle or BYI 02960 (50, 150 or 500 mg/kg/day) daily by dermal application for at least 28 consecutive days (minimum of 6 h/day) and euthanized one day following the last dose. The toxicological response of the rat was principally characterized by non-adverse decreases in food consumption in female rats at 500 mg/kg/day in the first two weeks and at 50 mg/kg/day in the second week and mild decreases in absolute and relative liver weights in males with no clinical pathology or micropathology correlates. The No-adverse-effect-level in this study was 500 mg/kg/day for both male and female rats.

Table 5.3-01: Summary of short-term toxicity of BYI 02960

Type of study (Document N°) Concentrations in feed	NOEL/NOAEL		LOAEL		Adverse effects at LOAEL
	ppm	mg/kg/d	ppm	mg/kg/d	
28-day rat study (M-283421-02-2) 0, 75, 200 & 350 mg/kg/day	-	75	-	200	Liver: centrilobular hepatocellular hypertrophy, both sexes Thyroid: Minimal diffuse follicular cell hypertrophy in males only at 200 mg/kg/day
28-day rat study (M-297120-01-2) 0, 500 & 5000 ppm	500	33.6	5000	385	Liver: slight to moderate diffuse centrilobular hepatocellular hypertrophy Thyroid: Minimal to slight diffuse follicular cell hypertrophy Decreased T4, increased TSH, BROD and UDPGT inductions
90-day rat study (M-329048-03-2) 0, 100, 500 & 2500 ppm	500	30/38	2500	156/186	Liver: centrilobular hepatocellular hypertrophy in both sexes Thyroid: follicular cell hypertrophy in males only
28-day mouse study (M-294820-01-2) 0, 300, 600 & 1200 ppm	960 to 1080	166 to 186	>960 to 1080	>166 to 186	Only slight body weight decrease
90-day mouse study (M-328668-03-2) 0, 100, 500 & 2500 ppm	500	80.6/98.1	2500	407/473	Liver: increased diffuse hepatocellular vacuolations Kidney: decreased multifocal/diffuse Corticoepithelial vacuolation :
28-day dog study (M-312461-01-3) 0, 500, 2000 & 4000 ppm	2000	62/77	4000	118/131	Liver: centrilobular glycogen accumulation decreased in incidence and/or severity
90-day dog study (M-369978-01-2) 0, 400, 1200 & 3600/2400 ppm	400	12/12	1200	33/41	Liver: increased absolute and relative weight in both sexes; brown pigment in Kupffer cells in females (high dose) Kidney: increased relative weights in both sexes Skeletal muscle: myofiber atrophy/degeneration in both sexes
1 year dog study (M-425272-01-2) 0, 150, 300, 1000 ppm	300	7.8/7.8	1000	28.1/28.2	Minimal to slight degeneration of skeletal muscle (gastrocnemius and biceps femoris) in both sexes
28-day dermal rat study (M-432336-01-1) 50, 150, 500 mg/kg/d	-	500	-	> 500	Non-adverse decreases in food consumption in females and mild decreases in absolute and relative liver weights in males

KIIA 5.3.1 - Oral 28-day toxicity

Report:	**KIIA 5.3.1/01, [redacted] A.; 2007**
Title:	**BYI 02960, Exploratory 28-day toxicity study in the rat by gavage**
Report No & Document No	SA 06075; M-283421-02-2
Guidelines:	Not applicable (only preliminary and explorative study design)
GLP	Study not performed under GLP, but laboratory GLP-certified

Executive Summary

The potential systemic toxicity of insecticide BYI 02960 (batch number NLL 7780-16-5: an orange, amorphous solid, 98.3% purity), was assessed after oral administration by gavage to groups of Wistar rats (5/sex/group) for at least 28 days at dose levels of 75, 200 and 350 mg/kg/day. A similarly constituted group of 5 rats per sex received the vehicle alone (corn oil supplemented with 10% ethanol and 10% water, v/v) at the same dosage volume of 5 mL/kg/day and served as a control. Animals were observed daily for mortality and clinical signs. Body weight and food consumption were recorded once weekly. Physical examinations were performed once during the acclimatization phase and weekly during the treatment period. Selected hematology and clinical chemistry parameters were assayed at the end of the study. At study termination (Study Day 30 or 31), all animals were necropsied, selected organs were weighed and a range of tissues were taken, fixed and examined microscopically. The remaining portions of the liver were homogenized for microsomal preparations in order to determine cytochrome P-450 isoenzyme profile.

At 350 mg/kg/day

Two females were found dead on Study Day 6 with no significant effects noted except for increased salivation on Study Day 2. No treatment related macroscopic or microscopic observations were noted for these animals. Increased salivation was observed in all animals in both sexes from Study Day 2 and on most days to the end of study. Mean body weight was slightly reduced in males by between 5% to 8% throughout the study period, in comparison with the controls (not statistically significant). In females, mean body weight was slightly reduced by 4% on Study Day 8 (not statistically significant). Mean food consumption was reduced by 17% in males and by 29% in females between Study Days 1 and 8, and by 8% (not statistically significant) in males between Study Day 8 and 15, compared to the controls. Clinical chemistry evaluations showed a decrease in total bilirubin, glucose concentrations, and an increase in triglyceride concentration in both sexes, whilst an increase in creatinine concentration and alanine aminotransferase and alkaline phosphatase activities was noted in females. Mean absolute and relative liver weights were 14 to 23% higher in males and 45 to 49% higher in females, compared to controls. Upon macroscopic examination, enlargement and prominent lobulation of the liver were observed in both sexes. Upon microscopic examination, slight to marked centrilobular hepatocellular hypertrophy was observed in the liver in both sexes. Minimal to slight diffuse follicular cell hypertrophy was observed in the thyroid gland in both sexes. Hepatocellular enzymatic activity assays showed an induction of BROD activity in both sexes, with no clear induction of PROD activity, indicating that BYI 02960 is an inducer of Cytochrome P-450 3A family.

At 200 mg/kg/day

One female, exhibiting increased salivation, was found dead on Study Day 2. No treatment-related macroscopic or microscopic observations were noted for this animal. Increased salivation was observed in all animals in both sexes from Study Day 2 and on most days through the end of study. Mean food consumption was reduced in females by 16% between Study Day 1 and 8, in comparison with the controls. In the clinical chemistry evaluation, decreases in total bilirubin concentration in both sexes and in glucose concentration in males were observed, and increases in triglyceride and creatinine concentrations, and alanine aminotransferase activity were observed in females. At final sacrifice, mean liver to body weight ratio was 10 to 11% higher in males and 21 to 25% higher in females as compared to controls. Upon macroscopic examination, enlargement and prominent lobulation were observed in males. Upon microscopic examination, minimal to moderate centrilobular hepatocellular hypertrophy in the liver was observed in both sexes. Minimal diffuse follicular cell hypertrophy was observed in the thyroid gland in males. Hepatocellular enzymatic activity assays showed an induction of BROD activity in males with no clear induction of PROD activity.

At 75 mg/kg/day

The only treatment-related effects were increased salivation, observed in all animals on several days from Study Day 13 onwards, and prominent lobulation of the liver, observed on one animal, without any associated microscopic findings. In the absence of any other changes, these findings were considered to be non-adverse.

In conclusion, the dose level of 75 mg/kg/day was considered to be a No Adverse Observed Effect Level (NOAEL) of BYI 02960 in both sexes in this study.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960

Description:	Orange amorphous solid
Lot/Batch:	NLL 7780-16-5
Purity:	98.3 %
CAS:	951659-40-8
Stability of test compound:	Stable in corn oil supplemented with 10% ethanol and 10% water (v/v) for 28 days at ambient temperature

2. Vehicle and /or positive control: Corn oil supplemented with 10% ethanol and 10% water (v/v)

3. Test animals:

Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age:	6 weeks approximately
Weight at dosing:	189 to 208 g for the males - 162 to 188 g for the females
Source:	[redacted] France
Acclimation period:	7 days

Diet:	Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 55 ± 15% Air changes: Approximately 10 to 15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates

02 August, 2006 to 08 September, 2006 at [redacted], France.

2. Animal assignment and treatment

There were 5 animals of each sex per dose group. Animals were assigned to dose groups using a randomization by weight. BYI 02960 was administered by gavage for at least 28 days to Wistar rats at the following doses - 0, 75, 200 and 350 mg/kg bw/day. A similarly constituted group of 5 rats per sex received the vehicle alone (corn oil supplemented with 10% ethanol and 10% water, v/v) at the same dosage volume of 5 mL/kg/day and acted as a control. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

3. Substance formulations and analysis

The dosing formulations were prepared by suspending the test substance (w/v) in corn oil supplemented with 10% ethanol and 10% water (v/v), to produce the required dosing concentrations. When not in use, the formulations were stored at room temperature. Four formulations were prepared for the study at each concentration.

The stability of the test substance in vehicle was checked in study SA 06225 at 1 and 100 g/l for 28 days at room temperature. The homogeneity of BYI 02960 in vehicle was verified on the first formulation for the lowest and highest concentrations to demonstrate adequate formulation procedures. Levels of the test substance in vehicle were verified for each formulation at each concentration. The mean values obtained from the homogeneity checks were used as measured concentrations. Homogeneity and concentration checks of BYI 02960 in dosing formulations were considered to be acceptable as they were in the range of 91 to 102% of nominal concentration and thus within the in-house acceptable range of 90 to 110% of nominal concentration.

Table 5.3.1-01 Study design

Test Group	Dose levels mg/kg/day	Animals assigned	
		Male	Female
1	0	5	5
2	75	5	5
3	200	5	5
4	350	5	5

4. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods

1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed once during the acclimatization phase and at least weekly during the treatment period.

2. Body weight

Body weights were recorded once during the acclimatization phase, on the first day of test substance administration, then at weekly intervals throughout the treatment and before necropsy.

3. Food consumption and compound intake

The weight of food supplied and that remaining at the end of the food consumption period were recorded weekly for all animals.

4. Clinical chemistry

On study Days 30 or 31, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane [redacted], France). Blood was collected on EDTA for hematology, lithium heparin for plasma chemistry and on sodium citrate for coagulation parameters.
The following haematology parameters were assayed using a Advia 120 (Bayer Diagnostics, [redacted], France): red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count. A blood smear was prepared and stained with Wright stain. It was examined when results of Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL 3000 (Instrumentation Laboratory, Paris, France).

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total protein, albumin, total cholesterol and triglycerides concentrations and aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed using an Advia 1650 (Bayer Diagnostics, [redacted], France).

5. Sacrifice and pathology

On Study Days 30 or 31, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane). An approximately equal number of animals randomly distributed amongst 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, all major organs, tissues and body cavities.

Adrenal gland, brain, kidney, liver, ovary, pituitary gland, spleen, testis, epididymis and thyroid gland (with parathyroid) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

The following organs or tissues were sampled: adrenal gland, epididymis, Kidney, Liver, Ovary, Pituitary gland, Spleen, Testis, Thyroid gland (with parathyroid gland), Uterus, Vagina, Macroscopic findings.

Samples were fixed by immersion in 10% neutral buffered formalin with the exception of epididymis and testis, which were fixed in Davidson's fixative. All the above mentioned samples were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared for all the organs from all the animals in all groups.

Histopathological examinations of all the above mentioned organs (except the parathyroid gland) were performed on all the animals in all dose groups.

6. Hepatotoxicity testing

At final necropsy, the remaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profiles to check the hepatotoxic potential of the test substance.

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. One quantification was performed for each sample.

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using the following substrates:

- benzoxyresorufin (BROD),
- ethoxyresorufin (EROD),
- pentoxyresorufin (PROD)

and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate). Ethoxyresorufin is a highly selective substrate for the isoform 1 A, the isoform 2B metabolizes preferentially the O-dealkylation of pentoxyresorufin, while the benzoxyresorufin O-debenzylation is mainly metabolized by the isoform 3A. Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37 °C.

Individual samples were prepared to follow the hydroxylation of lauric acid by the isoform 4A over a period of 10 minutes at 37 °C. Two replicates of each incubation mixture were collected. One replicate was analyzed, the other one was stored frozen. 12-hydroxylauric acid was extracted using diethylether from liver microsome incubation mixtures pooled by groups. After evaporation, the residue was dissolved in acetonitrile and analyzed by HPLC using fluorescence detection and pre-column derivatization. Quantification was performed by internal standardization with octanoic acid.

II. Results and discussion

A. Observations

1. Clinical signs

Increased salivation was observed in all animals at all dose levels tested. At 350 and 200 mg/kg/day, it was observed from Study Day 2 and on most days through the end of the study. At 75 mg/kg/day, it was observed on several occasions from between Study Day 13 and 24.
The few other clinical signs observed occurred in isolation at the mid or low dose level and are therefore considered not to be treatment-related.

2. Mortality

At 350 mg/kg/day, two female rats were found dead on Study Day 6. At 200 mg/kg/day, one female rat was found dead on Study Day 2. The only treatment-related clinical sign observed for these animals was increased salivation on Study Day 2. All macroscopic or microscopic observations were considered to be incidental and not treatment-related. The cause of death was unknown.

B. Body weight and body weight gain

At 350 mg/kg/day, mean body weight was slightly reduced in males by between 5% and 8% (not statistically significant) throughout the study period, in comparison with the controls. A slight reduction in mean body weight gain (not statistically significant) was observed in males at the end of the first and second weeks of treatment.
In females, mean body weight was slightly reduced by 4% on Study Day 8 (not statistically significant).
At 200 and 75 mg/kg/day, mean body weight and mean body weight gain parameters were unaffected by treatment in either sex.

C. Food consumption

At 350 mg/kg/day, mean food consumption was reduced by 17% in males ($p < 0.01$) and by 29% in females ($p < 0.01$) between Study Day 1 and 8, and by 8% (not statistically significant) in males between Study Day 8 and 15, compared to the controls. Thereafter, mean food consumption was comparable to or slightly higher than the controls.
At 200 mg/kg/day, mean food consumption was unaffected by treatment in males. In females, mean food consumption was reduced by 16% between Study Day 1 and 8 ($p < 0.05$), in comparison with the controls. Thereafter, mean food consumption was comparable to or slightly higher than the controls.
At 75 mg/kg/day, mean food consumption was unaffected by treatment in either sex.

D. Blood analysis

1. Haematological findings

No treatment-related changes were noted for the parameters assayed at any dose level tested.

2. Clinical chemistry findings

Treatment-related changes were observed at 350 and/or 200 mg/kg/day and included lower total bilirubin, lower glucose and higher triglyceride concentrations in both sexes and higher creatinine concentration, higher alanine aminotransferase and higher alkaline phosphatase activities in females only.

Table 5.3.1-02: Significant changes in clinical chemistry

Clinical chemistry parameter changes (% change when compared to controls)								
Sex	**Male**				**Female**			
Dose level of BYI 02960 (mg/kg/day)	**0**	**75**	**200**	**350**	**0**	**75**	**200**	**350**
Total bilirubin concentration	1.2 -	0.7* (- 42%)	**0.6* (- 50%)**	**0.4** (- 67%)**	1.6 -	0.9 (- 44%)	**0.7* (- 56%)**	**0.5** (- 69%)**
Glucose concentration	5.42 -	4.60 (- 15%)	**3.92** (- 28%)**	**2.87** (- 47%)**	4.80 -	5.07 (+ 6%)	5.12 (+ 7%)	**3.57 (- 26%)**
Triglyceride concentration	1.14 -	1.50 (+ 32%)	1.32 (+16%)	**1.89 (+ 66%)**	0.45 -	0.46 (+ 2 %)	**0.87 (+ 93%)**	**1.51* (+ 236%)**
Creatinine concentration	50 -	51 (+ 2%)	54* (+ 8%)	53 (+ 6%)	51 -	56 (+ 10%)	**63** (+ 24%)**	**63* (+ 24%)**
Alanine aminotransferase activity	39 -	43 (+ 10%)	38 (- 3%)	46 (+ 18%)	33 -	35 (+ 6%)	**43** (+ 30%)**	**51** (+ 55%)**
Alkaline phosphatase activity	165 -	163 (- 1%)	166 (+ 1%)	165 (+ 0%)	95 -	110 (+ 16%)	96 (+ 1%)	**129* (+ 36%)**

* : $p \leq 0.05$ ** : $p \leq 0.01$

The statistically significant difference observed in total bilirubin at 75 mg/kg/day in males was considered not to be treatment-related as only one animal was affected (animal QT2M2739, with a total bilirubin concentration of 0.0 µmol/l, while the mean of the remainder of the group was 0.85 µmol/l). The slightly higher creatinine concentration noted at 200 mg/kg/day in males was considered incidental in absence of a similar change being observed at the higher dose level of 350 mg/kg/day. No other treatment-related change was noted for the parameters assayed.

E. Sacrifice and pathology

1. Organ weight

Mean terminal body weight was marginally lower at 350 mg/kg/day in males when compared to controls (- 7%, not statistically significant).

At 350 and 200 mg/kg/day, mean absolute and relative liver weights were higher in both sexes when compared to controls.

Table 5.3.1-03: Significant organ weight changes

Liver weight changes at terminal sacrifice (% change when compared to controls)								
Sex	**Male**				**Female**			
Dose level BYI 02960 (mg/kg/day)	**0**	**75**	**200**	**350**	**0**	**75**	**200**	**350**
Mean absolute liver weight (g)	10.92	9.97 (- 9%)	12.01 (+ 10%)	12.43 (+ 14%)	5.86	6.37 (+ 9%)	7.32 (+ 25%)	8.74** (+ 49%)
Mean liver to body weight ratio	3.095	2.913 (- 6%)	3.442 (+ 11%)	3.814* (+ 23%)	2.552	2.850 (+ 12%)	3.157* (+ 24%)	3.704 (+ 45%)
Mean liver to brain weight ratio	542.705	482.53 (- 11%)	602.567 (+ 11%)	625.666 (+ 15%)	315.092	328.833 (+ 4 %)	382.274 (+ 21%)	460.553** (+ 46%)

* : $p \leq 0.05$ ** : $p \leq 0.01$

All other organ weight differences were judged to be incidental in view of their individual variation.

2. Gross and histopathology

Three females, dosed at 350 mg/kg/day and one female dosed at 200 mg/kg/day were found dead before the end of the study. All macroscopic lesions observed at necropsy were considered to be incidental and not treatment-related. All the microscopic changes observed in the three decedent females were considered not to be treatment-related. As only selected organs were taken and observed, the cause of death was unknown.

At terminal sacrifice, enlargement and prominent lobulation were observed in the liver in both sexes at 350 mg/kg/day and in males at 200 mg/kg/day. These changes were correlated with microscopic centrilobular hepatocellular hypertrophy. In addition, one male animal treated at 75 mg/kg/day showed prominent lobulation of the liver, but this was without any associated microscopic findings. This finding was therefore considered not to be adverse.
Other macroscopic changes were considered as incidental and not treatment-related.

Table 5.3.1-04: Significant macroscopic findings

Incidence of macroscopic changes in the liver - terminal sacrifice								
Sex	**Male**				**Female**			
Dose level BYI 02960 (mg/kg/day)	**0**	**75**	**200**	**350**	**0**	**75**	**200**	**350**
Enlarged	0/5	0/5	1/5	3/5	0/5	0/5	0/4	3/3
Prominent lobulation	0/5	1/5	2/5	5/5	0/5	0/5	0/4	2/3

Microscopic effects of treatment with BYI 02960 were seen in the liver and the thyroid gland. Minimal to marked centrilobular hepatocellular hypertrophy was observed in the liver in both sexes at 350 and 200 mg/kg/day. These changes were considered to be treatment-related. Minimal to slight diffuse follicular cell hypertrophy was observed in the thyroid gland in both sexes at 350 mg/kg/day and in males at 200 mg/kg/day. These changes were considered to be treatment-related. The other histopathological findings were considered to be incidental and not treatment-related.

Table 5.3.1-05: Incidence and severity of significant microscopic findings

Sex	Male				Female			
Dose levels BYI 02960 mg/kg/day	**0**	**75**	**200**	**350**	**0**	**75**	**200**	**350**
Number of animals	**5**	**5**	**5**	**5**	**5**	**5**	**4**	**3**
Liver: centrilobular hypertrophy: diffuse								
Minimal	0	0	0	0	0	0	2	0
Slight	0	0	4	1	0	0	1	2
Moderate	0	0	1	3	0	0	0	1
Marked	0	0	0	1	0	0	0	0
Total	**0**	**0**	**5**	**5**	**0**	**0**	**3**	**3**
Thyroid gland: Follicular cell hypertrophy: diffuse								
Minimal	0	0	4	4	0	0	0	2
Slight	0	0	0	1	0	0	0	1
Total	**0**	**0**	**4**	**5**	**0**	**0**	**0**	**3**

F. Hepatotoxicity testing

Total cytochrome P-450 contents were very slightly increased in males at 200 and 75 mg/kg/day and in females at 350 and 200 mg/kg/day, when compared to the controls.

BROD activity was increased at 350 mg/kg/day by approximately twenty five fold in males and twenty eight fold in females. At 200 mg/kg/day, BROD activity was increased by twenty five fold in males and eight fold in females. At 75 mg/kg/day, BROD activity was slightly increased by eight fold in males. EROD activity had a tendency to be very slightly increased at 350 mg/kg/day (three fold in males and four fold in females) and at 200 mg/kg/day (four fold) in females. However, compared to the positive control β-naphthoflavone , the magnitude of the increase remained at a very low level. PROD activity was very slightly increased at 350 mg/kg/day in both sexes and at 200 mg/kg/day in males (four fold compared to the control values). However, compared to the positive control phenobarbital, the magnitude of the increase remained at a very low level. The lauric acid hydroxylation levels were not affected by treatment in either sex. Therefore, BYI 02960 appears to be an inducer of the Cytochrome P-450 3 A family.

III. Conclusions

The dose level of 75 mg/kg/day was considered to be a No Adverse Observed Effect Level (NOAEL) of BYI 02960 in both sexes in this study, as the clinical sign observed and the macroscopic observation were considered to be non-adverse.

Report:	KIIA 5.3.1/02, [redacted] M.; 2008
Title:	BYI 02960, Exploratory 28-day toxicity study in the rat by dietary administration
Report No & Document No	SA 07047; M-297120-01-2
Guidelines:	Not applicable (only preliminary and explorative study design)
GLP	Study not performed under GLP, but laboratory GLP-certified

Executive Summary

BYI 02960 (batch number NLL 7780-27-1: a beige powder, 99.7% purity) was administered continuously via the diet to groups of male Wistar rats (5 /group) for at least 28 days at nominal concentrations of 500 ppm (actual analyzed concentration of 410 ppm equivalent to 33.6 mg/kg body weight/day) and 5000 ppm corresponding to 385 mg/kg body weight/day. A similarly constituted group received untreated diet and served as a control.

At 5000 ppm

Mean body weight was reduced by between 17 and 19% throughout the study period in comparison with the controls. Mean body weight gain per day was markedly reduced between Study Day 1 and 8 (1 g/day compared to 7 g/day in the control; $p < 0.01$) and slightly between Study Day 8 and 15 (6 g/day compared to 8 g/day in the control). Overall, mean cumulative body weight gain was reduced by 38% compared to the controls by Study Day 29. Mean food consumption was consistently reduced by between 12 and 39% ($p < 0.01$) for each measured interval, the effect being more pronounced between Study Day 1 and 8.

Statistically significant lower glucose (- 46%; $p < 0.01$) and total bilirubin (- 73%; $p < 0.05$) concentrations and higher urea (+ 37%; $p < 0.01$) and total cholesterol (+ 41%; $p < 0.05$) concentrations were observed at the end of the study.

Hormone analysis showed an increase in TSH (+ 81%) and a slight decrease in T4 (- 19%) in the plasma (neither being statistically significant).

The liver was affected by treatment with increase in mean relative weight, prominent lobulation and slight to moderate diffuse centrilobular hepatocellular hypertrophy. Minimal to slight diffuse follicular cell hypertrophy was observed in the thyroid gland of all animals.

The assessment of the P-450 isoenzyme activities at the end of the 28-day treatment period revealed increases in mean BROD and mean UDPGT activities.

At 500 ppm

No treatment related findings were observed at this dose level.

In conclusion, the dose level of 500 ppm (actual analyzed dose level of 410 ppm, which equates to 33.6 mg/kg/day BYI 02960) was considered to be the No Observed Effect Level (NOAEL) in this study.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	NLL 7780-27-1
Purity:	99.7%
CAS:	951659-40-8
Stability of test compound:	Stable in rodent diet at 5000 ppm over a 34-day frozen period followed by 8 days at ambient temperature; at 500 ppm when stored frozen for 31 days followed by 11 days at ambient temperature, some samples were slightly outside the house range
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj: WI(IOPS)HAN)
Age:	6 weeks approximately
Weight at dosing:	201 to 214 g for the males
Source:	[redacted] France
Acclimation period:	7 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 55 ± 15% Air changes: Approximately 15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates

04 June to 12 July 2007, [redacted], France.

2. Animal assignment and treatment

There were 5 male rats per dose group. Animals were assigned to dose groups using a randomization by weight. BYI 02960 was administered in the diet for at least 28 days to Wistar rats at the following doses - 0, 500 (actual analyzed concentration of 410 ppm) and 5000 ppm (equating approximately to 33.6 and 385 mg/kg/day). A negative control group received plain diet. Animal housing and husbandry were in

accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

3. Diet preparation and analysis

BYI 02960 was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. The stability of the test substance in the diet was determined on a pre-study mix at concentrations of 50, 500, 5000 and 20000 ppm for a time which covered the period of usage and storage for the study. The results showed that BYI 02960 was stable in ground rodent diet formulations at the dose level of 20000 ppm for at least 46 days stored frozen followed by 10 days stored at room temperature and for at least 56 days stored at room temperature. At the dose level of 50 ppm BYI 02960 was stable in ground rodent diet formulations for at least 11 days stored at room temperature (100%) or for at least 31 days stored frozen (100%) or for at least 31 days stored frozen followed by 11 days stored at room temperature (85%). At the dose level of 500 ppm, BYI 02960 was stable in ground rodent diet formulations for at least 34 days stored frozen followed by 4 days stored at room temperature and for at least 34 days stored frozen. At the dose level of 5000 ppm, BYI 02960 was stable in ground rodent diet formulations for at least 34 days stored frozen followed by 8 days stored at room temperature and for at least 34 days stored frozen. As the stability of the BYI 02960 at 500 ppm was shown to be at 82% of the nominal concentration (out of the in-house acceptable range) after 34 days frozen storage and 8 days at room temperature, this value was taken into account to calculate the achieved dosage.

The homogeneity of BYI 02960 in the diet was verified for the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. Dietary levels of the test substance were verified for each concentration. Homogeneity and concentration checks of BYI 02960 in diet formulations were in the range of 97 and 99% of nominal concentration and thus within the in-house acceptable range of 85 to 115% of nominal concentration. The diet formulations were therefore considered acceptable for use in the current study.

Table 5.3.1-06: Study design

Test Group	Diet Concentration (ppm)	Animals assigned
		Male
1	0	5
2	500	5
3	5000	5

4. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a

Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods

1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed once during the acclimatization phase and at least weekly during the treatment period.

2. Body weight

Each animal was weighed at least weekly during the acclimatization period, on the first day of test substance administration, then at weekly intervals throughout the treatment period. Additionally diet fasted animals were weighed before scheduled necropsy.

3. Food consumption and compound intake

Food consumption was recorded weekly; the weekly mean achieved dosage intake for each week and for weeks 1 to 4 was calculated.

4. Clinical chemistry

On study day 30 prior to necropsy, blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of isoflurane. Blood was collected on lithium heparin for clinical chemistry and hormone analysis.

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total protein and albumin, total cholesterol, triglycerides, and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed were assayed using an Advia 1650 (Bayer Diagnostics, [redacted], France).

5. Hormone analysis

T4 and TSH levels were determined from plasma samples collected on all animals at terminal sacrifice using specific radio-immunoassay kits (supplied by Beckman Coulter for T4 and by GE Healthcare for TSH).

6. Sacrifice and pathology

On Study Day 30, all animals from all groups were sacrificed by exsanguination whilst under deep anesthesia (Isoflurane inhalation). Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically.

The following organs were weighed: Kidney, Liver, Testis, Thyroid gland (with parathyroid gland). Paired organs were weighed together. The same organs and macroscopic findings were sampled and

fixed by immersion in neutral buffered 10% formalin with the exception of testis that was fixed in Davidson's fixative. Histological sections were prepared for all animals in all groups and stained with hematoxylin and eosin. Histopathological examinations were performed on all tissues except for the parathyroid gland, for all animals.

7. Hepatotoxicity testing

At final necropsy, the remaining portions of the liver from all animals were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profiles to check the hepatotoxic potential of the test substance.

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. One quantification was performed for each sample.

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using the following substrates:

- benzoxyresorufin (BROD),
- ethoxyresorufin (EROD),
- pentoxyresorufin (PROD).

Ethoxyresorufin is a highly selective substrate for the isoform 1 A, the isoform 2B metabolizes preferentially the O-dealkylation of pentoxyresorufin, while the benzoxyresorufin O-debenzylation is mainly metabolized by the isoform 3A. Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37 °C.

Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate (method adapted from Zakim and Vessey (17)). The enzymatic kinetic (disappearance of the colored 4-nitrophenol) was followed at 405 nm during 3 min at 30 °C. Three replicates from each sample were assayed. Microsomes induced by well known reference compounds p-naphtoflavone and phenobarbital were measured at the same time as the study samples to have positive controls for each assay.

II. Results and discussion

A. Observations

1. Clinical signs of toxicity

There were no clinical signs observed during the study.

2. Mortality

There were no mortalities during the course of the study.

B. Body weight and body weight gain

At 5000 ppm, mean body weight was reduced by between 17 and 19% throughout the study period (the effect being statistically significant for the first 3 weeks of the study) in comparison with the controls. Mean body weight gain per day was reduced between Study Day 1 and 8 (1 g/day compared to 7 g/day in the control; $p < 0.01$) and between Study Day 8 and 15 (6 g/day compared to 8 g/day in the control; p

<0.01). Thereafter, mean body weight gain was similar to the controls. Overall, mean cumulative body weight gain was reduced by 38% (p <0.01) compared to the controls between Study Day 1 and 29.
BYI 02960 dietary administration at a nominal concentration of 500 ppm had no effect on body weight.

C. Food consumption and compound intake

At 5000 ppm, mean food consumption was reduced by between 12 and 39% (p < 0.01) for each measured interval, the effect being more pronounced between Study Day 1 and 8. At 500 ppm mean food consumption was unaffected by the treatment.
The mean achieved dietary intake of BYI 02960 expressed in mg/kg/day received by the animals during the study were as follows:

Table 5.3.1-07: Mean achieved dietary intake of BYI 02960 (Weeks 1 - 4)

500 ppm (nominal diet concentration*)	41.0 mg/kg/day (theoretical)
410 ppm (actual diet concentration**)	33.6 mg/kg/day (actual)
5000 ppm (nominal diet concentration)	385 mg/kg/day

* : Assuming 100% recovery if not stability issues
** : Based on actual recovery of 82%

D. Blood analysis

1. Clinical chemistry findings
Treatment-related changes were seen at 5000 ppm. Mean glucose and total bilirubin concentrations were significantly decreased whereas mean urea concentration was significantly increased. In addition, a slightly higher mean total cholesterol concentration was observed when compared to controls. No relevant changes were seen at 500 ppm.

Table 5.3.1-08: Significant clinical chemistry changes
[Mean ± standard deviation (% change when compared with controls)]

Dose level of BYI 02960 (ppm)	0	500	5000
Glucose (mmol/l)	6.91 ± 1.25	6.60 ± 1.08 (- 4%)	3.76 ± 0.37** (- 46%)
Total bilirubin (µmol/l)	1.1 ± 0.3	0.7 ± 0.4 (-36%)	0.3 ± 0.3* (- 73%)
Urea (mmol/l)	4.98 ± 0.76	4.82 ± 0.22 (- 3%)	6.83 ± 0.80** (+ 37%)
Total cholesterol (mmol/l)	1.43 ± 0.23	1.29 ± 0.21 (- 10%)	2.01 ± 0.49** (+ 41%)

* : p <0.05 ** : p <0.01

2. Hormone analysis
At 5000 ppm, a not statistically significant increase (+ 81%) in mean TSH levels was observed. Although not statistically significant, this increase was considered as treatment-related. At 500 ppm, no relevant change in TSH levels was observed.

At 5000 ppm, a not statistically significant decrease (- 19%) in mean T4 levels was observed. This change could be linked to the observed increase in mean TSH levels at the same dose level. At 500 ppm, no relevant change in T4 levels was observed.

Table 5.3.1-09: Mean hormone values and magnitude of increase relative to the controls

Dose level of BYI 02960 (ppm)	0	500	5000
T4 (nmol/L)	48.5 ± 9.2	58.7 ± 10.2 (+ 21%)	39.5 ± 6.6 (-19%)
TSH (ng/mL)	9.64 ± 4.77	11.23 ± 5.41 (+ 17%)	17.46 ± 5.38 (+ 81%)

E. Sacrifice and pathology

1. Organ weights

The mean terminal body weight was lower at 5000 ppm when compared to controls (-19%), though the effect was not statistically significant.

The mean liver and thyroid gland to body weight ratios were statistically significantly higher at 5000 ppm when compared to controls. All other organ weight differences were judged to be incidental in view of their individual variation.

Table 5.3.1-10: Significant organ weight changes at terminal sacrifice

Sex	Male		
Dose level of BYI 02960 (ppm)	**0**	**500**	**5000**
Liver			
Mean absolute liver weight (g)	9.79 ± 0.70	10.38 ± 0.26 (+ 6%)	11.25 ± 1.20 (+ 15%)
Mean liver to body weight ratio (%)	2.751 ± 0.127	2.873 ± 0.070 (+ 4%)	3.906 ± 0.405** (+ 42%)
Thyroid gland			
Mean absolute thyroid gland weight (g)	0.0171 ± 0.0012	0.0165 ± 0.0016 (-4%)	0.0182 ± 0.0005 (+6%)
Mean thyroid gland to body weight ratio (%)	0.00480 ± 0.00022	0.00457 ± 0.00044 (- 5%)	0.00632 ± 0.00017** (+ 32%)

** : p <0.01

2. Gross and histopathology

Prominent lobulation in liver was found in 4/5 males at 5000 ppm and in 2/5 males at 500 ppm. This was correlated to microscopic diffuse centrilobular hepatocellular hypertrophy only at the top dose and was considered to be treatment-related at 5000 ppm.

Treatment-related effects were found in liver and in the thyroid gland following exposure to 5000 ppm of BYI 02960.

In the liver, slight to moderate diffuse centrilobular hepatocellular hypertrophy was found in all animals at 5000 ppm. The grade of this effect was found to be slight in two animals and moderate in the three other ones. This finding was considered to be toxicologically relevant.

In the thyroid gland, minimal (2/5) to slight (3/5) diffuse follicular cell hypertrophy was observed in all the animals at 5000 ppm. This finding was considered to be dose related and toxicologically relevant.

F. Hepatotoxicity testing

At the end of the study, due to the breakdown of the ultra-centrifuge, the livers were weighed, frozen and stored at below -70 °C instead of being processed on the day of necropsy. This may have led to a denaturation of a part of the cytochrome P-450 contents and its transformation into non-active cytochrome P-420. For this reason, the hepatotoxicity may be under estimated.
BYI 02960 administration induced the following changes:

- no apparent change in total P-450 content at either dose level,
- no change in mean EROD activity at either dose level,
- no change in mean PROD activity at either dose level,
- a slight increase (+210% compared to the controls) in mean BROD activity at 5000 ppm only,
- a moderate increase (+11 1%) in mean UDPGT (4-nitrophenol) activity at 5000 ppm only.

Overall, the finding of no induction in EROD and PROD, with BROD slightly induced indicate that BYI 02960 at the dose level 5000 ppm, may be an inducer of the cytochrome P-450 3A family.

III. Conclusions

In rats receiving diet containing BYI 02960 for 28 days, treatment-related findings were observed at 5000 ppm consisting of reduced body weight and food consumption, decrease in glucose and total bilirubin concentrations and increase in urea and total cholesterol concentrations. Hormone analysis showed an increase in TSH and a slight decrease in T4. Target organs were the liver and the thyroid gland. Mean relative weights of both organs were higher compared to controls. Slight to moderate diffuse centrilobular hepatocellular hypertrophy in the liver was observed in all the animals. Minimal to slight diffuse follicular cell hypertrophy was observed in the thyroid in all animals. An increase in BROD and UDPGT activities was also observed at the end of the study.
No treatment related findings were observed at 500 ppm.

In conclusion, the dose level of 500 ppm (actual analyzed dose level of 410 ppm, which equates to 33.6 mg/kg/day BYI 02960) was considered to be the No Observed Effect Level (NOAEL) in this study.

Report:	**KIIA 5.3.1/03, [redacted] O.; 2007**
Title:	BYI 02960, Preliminary 28-day toxicity study in the mouse by dietary administration
Report No & Document No	SA 07013; M-294820-01-2
Guidelines:	Not applicable (only preliminary and explorative study design)
GLP	Study not performed under GLP, but laboratory GLP-certified

Executive Summary

BYI 02960 (batch number NLL 7780-27-1: a beige powder, 99.7% purity) was administered continuously via the diet to groups of C57BL/6J mice (5/sex/group) for at least 28 days at concentrations of 300, 600 and 1200 ppm (equating approximately to 50, 98 and 207 mg/kg body weight/day in males and 59, 122 and 240 mg/kg body weight/day in females). A similarly constituted group received untreated diet and served as a control.
Taking into consideration the available stability data, the actual concentration received by the treated animals is assumed to be between 80% and 90% of nominal concentrations.

At 1200 ppm
In males, mean body weight was slightly reduced by 6% at Study day 8 compared to the controls. Overall cumulative mean body weight gain was reduced by 15% between Study Days 1 and 29 compared to the controls. As the effect on body weight was slight and transient and in the absence of other findings, it was considered to be a non-adverse effect of treatment.
No treatment-related effects were observed in females.

At 600 and 300 ppm
No treatment-related effects were observed in either sex.

In conclusion, continuous dietary administration of BYI 02960 to the C57BL/6J mouse for at least 28 days resulted in a NOAEL in males and a NOAEL in females at a nominal concentration of 1200 ppm. However given the instability of BYI 02960 in rodent diet the actual concentration is considered to be in the region of 960 to 1080 ppm (166 to 186 mg/kg body weight/day for the males and 192 to 216 mg/kg body weight/day for the females, respectively).

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	NLL 7780-27-1
Purity:	99.7%
CAS:	951659-40-8
Stability of test compound:	Stable in rodent diet at 5000 ppm over a 34-day frozen period followed by 8 days at ambient temperature; Recovery rates were between 80% and 87% (18 days frozen +10 days at room temperature and 34 days frozen + 4 days at room temperature, at 50 and 500 ppm respectively)
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Mouse
Strain:	C57BL/6J mice
Age:	6 weeks approximately
Weight at dosing:	19.1 to 22.4 g for the males and 15.4 to 18.7 g for the females
Source:	[redacted] France
Acclimation period:	15 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 55 ± 15% Air changes: Approximately 15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design and methods

1. In life dates

11 April to 25 May, 2007 at [redacted] France.

2. Animal assignment and treatment

There were 5 male and 5 female mice per dose group. Animals were assigned to dose groups using a randomization by weight. BYI 02960 was administered in the diet for at least 28 days to C57BL/6J mice at the following doses - 0, 300, 600 and 1200 ppm (equating approximately to 50, 98 and 207 mg/kg body weight/day in males and 59, 122 and 240 mg/kg body weight/day in females). A negative

control group received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

3. Diet preparation and analysis

BYI 02960 was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. The stability of the test substance in the diet was determined on a pre-study mix at concentrations of 50, 500, 5000 and 20000 ppm for a time which covered the period of usage and storage for the study. The results showed that BYI 02960 was stable in ground rodent diet formulations at the dose level of 20000 ppm for at least 46 days stored frozen followed by 10 days stored at room temperature and for at least 56 days stored at room temperature. At the dose level of 50 ppm, BYI 02960 was stable in ground rodent diet formulations for at least 11 days stored at room temperature (100%) or for at least 31 days stored frozen (100%) or for at least 31 days stored frozen followed by 11 days stored at room temperature (85%). At the dose level of 500 ppm, BYI 02960 was stable in ground rodent diet formulations for at least 34 days stored frozen followed by 4 days stored at room temperature and for at least 34 days stored frozen. At the dose level of 5000 ppm, BYI 02960 was stable in ground rodent diet formulations for at least 34 days stored frozen followed by 8 days stored at room temperature and for at least 34 days stored frozen. As the stability of the BYI 02960 at 500 ppm was shown to be at 82% of the nominal concentration (out of the in-house acceptable range) after 34 days frozen storage and 8 days at room temperature and taking into consideration the available stability data, the actual concentration received by the treated animals was assumed to be between 80% and 90% of nominal concentrations.

No analysis was performed on the dietary formulations.

Table 5.3.1-11: Study design

Test Group	Diet Concentration (ppm)	Animals assigned	
		Male	Female
1	0	5	5
2	300	5	5
3	600	5	5
4	1200	5	5

4. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods

1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed once during the acclimatization phase and at least weekly during the treatment period.

2. Body weight

Each animal was weighed at least weekly during the acclimatization period, on the first day of test substance administration, then at weekly intervals throughout the treatment period. Additionally, diet fasted animals were weighed before scheduled necropsy.

3. Food consumption and compound intake

Food consumption was recorded weekly; the weekly mean achieved dosage intake for each week and for weeks 1 to 4 was calculated.

4. Clinical chemistry

On study day 30 prior to necropsy, blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of isoflurane. Blood was collected on lithium heparin for clinical chemistry and hormone analysis.

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total protein and albumin, total cholesterol, triglycerides, and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed were assayed using an Advia 1650 (Bayer Diagnostics, [redacted], France).

5. Sacrifice and pathology

On Study Day 30, all animals from all groups were sacrificed by exsanguination whilst under deep anesthesia (Isoflurane inhalation). Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically.

The following organs were weighed: Adrenal, brain, epididymis, kidney, liver, ovary, spleen, testis, uterus. Paired organs were weighed together. The following organs or tissues were sampled: Adrenal gland, epididymis, kidney, liver, ovary, spleen, testis, thyroid gland (with parathyroid gland), uterus, vagina, macroscopic findings; and fixed by immersion in neutral buffered 10% formalin with the exception of testis that was fixed in Davidson's fixative. Histopathological examinations were performed on all slides (except parathyroid gland) for all animals from control and high dose groups and for all animals found dead or killed moribund. Kidney, liver, thyroid gland and macroscopic findings of all animals were examined in the intermediate dose groups.

II. Results and discussion

A. Observations

1. Clinical signs of toxicity

There were no clinical signs observed during the study.

2. Mortality

There were no mortalities over the course of the study.

B. Body weight and body weight gain

At 1200 ppm, mean body weight in males was slightly reduced by 6% at Study Day 8 compared with the controls, though the effect was not statistically significant. Mean body weight gain/day was - 0.03 g ($p < 0.05$) compared with 0.14 g in the controls between Study Days 1 and 8. Thereafter, body weight evolution was comparable to the controls. Overall mean cumulative body weight gain between Study Days 1 and 29 was 15% lower than in the control group, but was not statistically significant. There was no effect on body weight or body weight gain in males administered 600 or 300 ppm diet.

For females, no effect on mean body weight or body weight gain was noted at any dose level.

C. Food consumption and compound intake

At 1200, 600 and 300 ppm, no effect on mean food consumption was noted in either sex.
The mean achieved dose levels of BYI 02960 expressed in mg/kg/day received by the animals during the study were as follows:

Table 5.3.1-12: Mean achieved dietary intake of BYI 02960 (Weeks 1 - 4)

Concentration (ppm)	Male mg/kg/day	Female mg/kg/day
300	50 (40*)	59 (47*)
600	98 (78*)	122 (98*)
1200	207 (166*)	240 (192*)

* : Based on actual recovery of 80%

D. Blood analysis

1. Clinical chemistry findings

When compared to the controls, a marginally higher alanine aminotransferase (+43%, $p < 0.01$) and alkaline phosphatase (+21%, $p < 0.05$) activities were observed in females at 1200 ppm. However in view of the variation of the individual values these changes were considered not to be treatment-related. No treatment-related changes were noted in males.

E. Sacrifice and pathology

1. Organ weights

There was no relevant change in mean terminal body weights when compared to controls.
All organ weight differences were judged to be incidental.

Lower epididymis weights were found in treated animals when compared to controls but this change was considered not to be relevant since it was not dose-related and not associated with relevant histological findings.
Mean absolute and relative spleen weights were statistically significantly higher in males at 300 ppm when compared to controls, but this change was considered not to be relevant since it was not dose-related.

2. Gross and histopathology
All macroscopic and microscopic changes were considered as incidental and not treatment-related.

III. Conclusions

BYI 02960 administered to mice for at least 28 days induced a slight body weight reduction in males at 1200 ppm.

In conclusion, the NOAEL in either sex was set at a nominal concentration of 1200 ppm. However given the instability of BYI 02960 in rodent diet the actual concentration was considered to be in the region of 960 to 1080 ppm (166 to 186 mg/kg body weight/day for the males and 192 to 216 mg/kg body weight/day for the females, respectively).

Oral 28-day toxicity in the dog

Report:	**KIIA 5.3.1/04 [redacted] M.; 2008**
Title:	BYI 02960, Preliminary 28-day toxicity study in the dog by dietary administration
Report No & Document No	SA 07290 M-312461-01-3
Guidelines:	Not applicable (only preliminary and explorative study design)
GLP	Study not performed under GLP, but laboratory GLP-certified

Executive Summary

BYI 02960 (batch number NLL 7780-44-6: a dark pink powder, 99.5% purity) was administered to beagle dogs of both sexes by the dietary route. Groups of two males and two females received BYI 02960 mixed in their diet at concentrations of 500, 2000 or 4000 ppm (equating approximately to 16, 62, 118 mg/kg body weight/day in males and 18, 77, 131 mg/kg body weight/day in females) for at least 28 days. A similarly constituted group of 2 males and 2 females received untreated diet, and served as a control.

At 4000 ppm
There was an overall body weight loss observed in one male whilst the body weight of the other male remained static. One female had an overall body weight gain of 0.7 kg whilst the body weight of the other female remained static. The control animals showed an overall gain of between 0.5 and 1.1 kg. Lower food consumption was observed in both male and female animals compared to the controls. Hematology assessment revealed a slightly increased platelet count in both females and in 1/2 males. In isolation this treatment-related change was not considered to be adverse. In the liver, centrilobular glycogen accumulation was decreased in incidence and/or severity in both sexes. This was considered to be a treatment-related but not adverse effect.

At 2000 ppm
Hematology assessment revealed a slightly increased platelet count in 1/2 females. In isolation this treatment-related changes was considered not to be adverse. In the liver, centrilobular glycogen accumulation was decreased in incidence and/or severity in males, which was considered to be treatment-related but not adverse effect.

At 500 ppm
None of the parameters evaluated were affected by the treatment at this dose level in either sex.
In conclusion, a dietary level of 2000 ppm (equating to 62 mg/kg/day in males and to 77 mg/kg/day in females) was considered to be a NOAEL in both sexes.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Pink powder
Lot/Batch:	NLL 7780-44-6
Purity:	99.5%
CAS:	951659-40-8
Stability of test compound:	Stable in diet at 4000 ppm over 48 days of storage at ambient temperature and at 500 ppm over 38 days of storage at ambient temperature
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Dog
Strain:	Beagle
Age:	8 months approximately
Weight at dosing:	6.7 to 9.0 kg for males and 5.2 to 7.0 kg for females
Source:	[redacted]
Acclimation period:	21 days
Diet:	Certified canine meal 125C3-P1 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France); Approximately 330 grams of diet moistened with 470 mL of water at the time of distribution presented to the animals for 1.5 hours/day; time period extended where necessary for the first few days after arrival
Water:	Tap water, *ad libitum*
Housing:	Animals were caged individually in stainless steel runs with a floor surface area of1.2 m^2
Environmental conditions:	Temperature: 19.5 ± 1.5 °C Humidity: 55 ± 15 % Air changes: Approximately 14 to 15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (6 am - 6 pm)

B. Study Design

1. In life dates

16 April to 05 June 2008 at [redacted], France.

2. Animal assignment and treatment

Prior to initiation of test compound administration, animals were allocated to dosage groups in order to ensure a similar body weight distribution among groups of each sex, whilst ensuring full siblings were

not placed in the same treatment group. Groups of two males and two females received BYI 02960 mixed in their diet at concentrations of 500, 2000 or 4000 ppm (equating approximately to 16, 62, 118 mg/kg body weight/day in males and 18, 77, 131 mg/kg body weight/day in females) for at least 28 days. A similarly constituted group of 2 males and 2 females received untreated diet, and acted as a control.

Table 5.3.1-13: Study design

Test group	Concentration in diet (ppm)	Dose per animal (average)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	2	2
2	500	16	18	2	2
3	2 000	62	77	2	2
4	4 000	118	131	2	2

3. Diet preparation and analysis

The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. The appropriate amount of test substance was incorporated into the ground diet (w/w) to provide the required dietary concentrations of 500, 2000 and 4000 ppm (theoretically corresponding to approximately 21, 85 and 169 mg/kg body weight/day, respectively, based on a daily weight of diet of 330 g and a mean body weight of 7.8 kg). For the two highest concentrations, one preparation (Fl) was prepared to provide the amount of treated diet required for the study. For the lowest concentration of 500 ppm, a second preparation (Flbis) was prepared due to unacceptable results concerning the concentration of the first formulation of 500 ppm.

The stability of BYI 02960 in the diet at 500 and 4000 ppm was determined after a minimum of 28 days under frozen storage conditions followed by 10 days at room temperature or after a minimum of 38 days of storage at room temperature.

The homogeneity of the test substance in the diet was verified before the start of the study at 500 and 4000 ppm on the preparations (Fl and Flbis) to demonstrate adequate formulation procedures.

The dietary level of the test substance in the diet was verified at each concentration on each preparation. BYI 02960 formulation in diet at 4000 ppm was found to be stable after at least 48 days of storage at room temperature and after 38 days of frozen storage followed by 10 days at room temperature. BYI 02960 formulation in diet at 500 ppm was found to be stable after at least 38 days of storage at room temperature and after 28 days of frozen storage followed by 10 days at room temperature. Results of homogeneity check or concentration of BYI 02960 were within the range of 88 to 96% of the nominal concentration. Hence all values were within the in-house range of 85 to 115% of the nominal concentration. The preparations were therefore considered acceptable for use on the current study.

4. Statistics

Due to the small number of animals per sex present in each group (n = 2), mean values and standard deviations were not calculated for the quantitative parameters with the exception of the achieved intake values, where individual and mean values are presented. Where applicable, the results obtained at the end of the treatment period were compared individually for each animal with the pre-study values, each animal serving as his own control.

C. Methods

1. Observations

Each animal was checked for ill-health, moribundity and mortality twice daily or once daily on weekends and public holidays. Any animal suffering from severe distress, in moribund condition or considered unlikely to survive was sacrificed and necropsied.

2. Body weight

Body weights were measured prior to feeding at least weekly during the acclimatization phase and treatment period, and before final necropsy.

3. Food consumption and compound intake

Food intake was measured for a minimum of five consecutive days before start of treatment. The weight of food supplied to each animal and that remaining were recorded daily throughout the treatment period. From these records, the mean weekly consumption was calculated for each dog. Food spillage was also recorded. The individual and group mean achieved dosage for each sex, expressed as mg/kg body weight/day, was calculated for each week and the overall mean subsequently derived for Weeks 1 to 4.

4. Clinical and physical examination

Observed clinical signs were recorded at least once daily throughout the study. A detailed physical examination was performed on all dogs at the end of the treatment period. The physical examination included but was not necessarily restricted to the following examinations: fur and skin, eyes, ears, teeth, gum, mucous membranes, rectal temperature, gait, stance, general behavior, chest including heart and respiratory rate, abdomen (including palpation, external genitalia and mammary glands.

The following parameters were also evaluated during the physical examination:

- Mental state: Level of consciousness, behavioral change
- Posture
- Gait and motor function
- Muscle tone
- Postural reactions: Placing (tactile and visual), Conscious propioceptive positioning (knuckling), Hopping and Wheelbarrowing
- Spinal nerve reflexes: patellar, withdrawal (flexor), pelvic and thoracic limb, perianal, panniculus
- Sensation: superficial pain or deep pain
- Cranial nerve reflexes: general examination of the head, direct papillary light, indirect papillary light, palpebral blink or cornea, menace.

5. Ophthalmic examination

During the acclimatization phase all animals were subjected to an ophthalmological examination after installation of an atropinic agent (Mydriaticum®, Merck-Sharp and Dohme). Each eye was examined by means of an indirect ophthalmoscope. At the end of treatment, all surviving animals were re-examined. In the case of treatment-related effects being identified, photographic records were made of the affected animals.

6. Clinical chemistry

On pre-test Days 13 or 14 and on study Day 27, blood samples were taken from all animals in all groups by puncture of the jugular vein. Blood was collected on EDTA for hematology, on heparin lithium (for plasma) and clot activator (for serum) for clinical chemistry and sodium citrate for coagulation parameters.

Red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count were assayed using an Advia 120 (Siemens, Eragny, France). A blood smear was prepared and stained using May-Grünwald-Giemsa method. It was examined when the results of Advia 120 determinations were abnormal. Prothrombin time and activated partial thromboplastin time were assayed on an ACL Elite Pro (Instrumentation Laboratory, Paris, France).

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus concentrations, and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed on plasma samples, total protein and albumin concentrations were assayed on serum samples using an Advia 1650 (Siemens, Eragny, France). Globulin concentrations and albumin/globulin ratio values were calculated.

7. Urinalysis

On pre-test Day 14 and on study Day 28 in the morning, overnight urine samples were collected from all animals in all groups. Access to water was not restricted during urine collection. Any significant change in the general appearance of the urine was recorded. The urine volume was measured. pH and specific gravity were assayed.

Urinary refractive index was measured using a RFM320 refractometer (Bioblock Scientific, Illkirch, France); pH was assayed using a Clinitek 500 and Multistix dipsticks (Siemens, Eragny, France). Glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen were assayed using a Clinitek 500 and Multistix dipsticks (Siemens, Eragny, France). Microscopic examination of the urinary sediment was performed after centrifugation of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was recorded.

8. Sacrifice and pathology

On study Days 29 and 30, all animals from all groups were tranquilized by intramuscular injection of acepromazine (0.5 mL/10 kg body weight) and sacrificed by exsanguination under deep anesthesia (pentobarbital, intravenous injection of approximately 60 mg/kg body weight). The animals were diet fasted prior to sacrifice.

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices, all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically.

Adrenal gland, brain, epididymis, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed fresh. Paired organs were weighed together.

The following organs or tissues were sampled: adrenal gland, aorta, bone (sternum, rib), bone marrow, brain, epididymis, oesophagus, eye and optic nerve, Fallopian tube, heart, intestine (duodenum,

jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver with gall bladder, lung, lymph nodes, mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, salivary gland, sciatic nerve, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, testis, thymus, thyroid gland (with parathyroid), trachea, urinary bladder, uterus (with cervix), ureter and vagina.
Samples were fixed by immersion in 10% neutral buffered formalin with the exception of the eye, optic nerve, epididymis and testis which were fixed in Davidson's fixative. All of the above mentioned samples, with the exception of larynx/pharynx, were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared from all the animals in all groups. Histotechnology was performed in-house. Histopathological examinations were performed on all animals from all groups.

II. Results and discussion

A. Observations

1. Mortality
No animals were found dead or sacrificed *in-extremis* in this study.

2. Clinical signs of toxicity
At 4000 ppm, one female was in oestrus between Study Day 15 to 22 with genital discharge.
At 2000 and 500 ppm, no clinical signs or changes at physical examination were noted in either sex.
Rectal temperature recording in all animals revealed no treatment-related changes.

B. Body weight and body weight gain
At 4000 ppm there was an overall body weight loss of 0.2 kg in one male whilst the body weight of the other male remained static. One female had an overall body weight gain of 0.7 kg whilst the body weight of the other female remained static. The control animals showed an overall gain of between 0.5 and 1.1 kg.
There was no treatment-related effect on body weight parameters at 2000 and 500 ppm in both sexes.

C. Food consumption and compound intake
At 4000 ppm, lower food consumption was observed in both male and both female animals compared to the controls. At 2000 ppm, food consumption was slightly lower than controls for both male and female animals, but was similar to their own pre-study values. At 500 ppm, the two females showed a decrease in food consumption during the three first weeks of the study compared to the controls, but the values were similar or higher than their own pre-study values.

The mean achieved dosage intake per group was: 16.1, 61.8 and 118.3 mg/kg/day in males and 17.8, 76.8 and 130.5 mg/kg/day in females.

D. Ophthalmic examination
No treatment-related ocular abnormalities were observed at ophthalmic examination.

E. Blood analysis

1. Haematological findings

The slightly increased platelet counts were observed in both females (+43 and +36%, relative to their own pre-study value) and in one of the two males at 4000 ppm (+30%, relative to its own pre-study value) and in one of the two females at 2000 ppm (+28%, relative to its own pre-study value). In isolation, these treatment-related changes were not considered to be adverse.

2. Clinical chemistry findings

At 4000 ppm, an increase was observed in creatinine concentration in one female (+31%, relative to its own pre-study value). As no relevant histopathological finding was noted in the kidneys this change was not considered to be treatment-related.

F. Urinalysis

No clear treatment-related change was noted in the parameters assayed in the study.

G. Sacrifice and pathology

1. Organ weight

There were no relevant changes in mean terminal body weights for either sex when compared to controls. All organ weight differences were judged to be incidental in view of their individual variation.

2. Gross and histopathology

Enlarged thyroid glands were noted in 2/2 females at 4000 ppm but this change was not considered to be treatment-related as there was no effect on thyroid weight and there were no microscopic findings for the thyroid.

In the liver, centrilobular glycogen accumulation was decreased in incidence and/or severity at 4000 and 2000 ppm in males and at 4000 ppm in females. This finding was considered to be treatment-related, but not adverse.

Table 5.3.1-14: Incidence and severity of microscopic changes in the liver

Sex	Male				Female			
Dose (ppm)	0	500	2,000	4,000	0	500	2,000	4,000
Number examined	2	2	2	2	2	2	2	2
Hepatocellular glycogen accumulation: centrilobular: diffuse								
Minimal	0	0	1	0	2	2	2	0
Slight	2	2	0	0	0	0	0	0
Total	**2**	**2**	**1**	**0**	**2**	**2**	**2**	**0**

III. Conclusions

In conclusion, a dietary level of 2000 ppm (equating to 62 mg/kg/day in males and to 77 mg/kg/day in females) was considered to be a No Observed Adverse Effect Level (NOAEL) in both sexes.

KIIA 5.3.2 - Oral 90-day toxicity (rodents)

Oral 90-day toxicity in the rat

Report:	**KIIA 5.3.2/01 [redacted] M.; 2009**
Title:	BYI 02960, 90-day toxicity study in the rat by dietary administration
Report No & Document No	SA 07294; M-329048-03-2
delines:	OECD 408 (1998); EEC Directive 2001/59/EC, Method B.26 (August, 2001) EPA Health Effects Test Guideline (OPPTS 870.3100; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
P	Yes (certified laboratory)

Executive Summary

BYI 02960 (batch number NLL 7780-44-6: a pink powder, 99.5% w/w purity) was administered continuously via dietary administration to separate groups of Wistar rats (10/sex/group) at concentrations of 100, 500 and 2500 ppm (equating to approximately 6.0, 30.2, 156 mg/kg body weight/day in males and 7.6, 38.3, 186 mg/kg body weight/day in females) for at least 90 days. A similarly constituted group of 10 males and 10 females received untreated diet and acted as a control. An additional 10 animals per sex were fed control or high dose test diet for at least 90 days and subsequently fed control diet and observed for reversibility or persistence of toxic effects after a post-treatment recovery period of at least 28 days.

<u>At 2500 ppm</u>

At 2500 ppm, a lower body weight was observed in both sexes throughout the study. A reduced mean body weight gain/day was observed in males at several occasions during the first two months of the study, whereas it was only noted during the first week of the treatment for female rats. At the end of the treatment period (Study Day 92), the mean body weight was 6% lower than the controls for males and females. Throughout the recovery phase of the study the mean body weight of males and females remained lower than the control group. The body weight gain of both males and females was higher than controls by 220 and 160% during the first week of the recovery phase. Thereafter, the mean body weight gain was comparable to or higher than the controls throughout the recovery phase of the study. A slight reduction in mean food consumption (17%) was observed for males during the first four days of the study and thereafter on several occasions (approximately 5% from Study Day 71 until the end of the main study phase). In females, a reduction in mean food consumption was observed from the first week of the study until Study Week 7 (between 9% and 29%). Thereafter, there was no evidence of a treatment-related effect on food consumption.

Hematological evaluation revealed a higher mean platelet count in females when compared to the control group (+15%). In addition, mean total bilirubin and glucose concentrations were slightly lower in both sexes and mean total cholesterol and triglycerides concentrations were slightly higher when compared to the controls. The change observed for total bilirubin was considered to be partially reversible in females, at the end of the recovery phase, as the mean concentration remained slightly lower. The other treatment-related changes were considered to be reversible as no relevant difference was noted after the recovery period.

At necropsy, the mean terminal body weights of treated males and females were lower compared to controls animals (-7% and -6% respectively). Mean liver to body weight ratio was statistically higher in

both sexes when compared to the controls. Mean thyroid gland to body weight ratio was statistically significantly higher in males when compared to controls. Enlarged liver was observed in some animals. Minimal to slight centrilobular hepatocellular hypertrophy was observed in both sexes. Dark thyroid gland at necropsy and microscopic minimal follicular cell hypertrophy were observed in some males. At the end of the recovery phase, no treatment-related findings were observed in the liver and the thyroid gland. All of the treatment-related findings observed at the end the treatment period were therefore considered to be reversible.

At 500 ppm
At 500 ppm, a reduced mean body weight gain/day was observed in females during the first week and the last week of the study. The overall mean body weight gain was 12% lower than the controls in females at the end of the treatment period. In the absence of any other changes in the parameters assessed, this finding was considered not to be adverse.

At 100 ppm
No toxicologically relevant changes were noted.

In conclusion, a dose level of 500 ppm of BYI 02960 represented a No Observed Adverse Effect Level (NOAEL) for both sexes (approximately 30.2 mg/kg/day for males and 38.3 mg/kg/day for females).

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Pink powder
Lot/Batch: NLL-7780-44-6
Purity: 99.5%
CAS: 951659-40-8
Stability of test compound: Stable in rodent diet at 2500 ppm after 102 days of storage at ambient temperature; at 20 ppm when stored frozen for 81 days followed by 10 days at ambient temperature

2. Vehicle and /or positive control: None

3. Test animals:
Species: Rat
Strain: Wistar Rj: WI (IOPS HAN)
Age: 6 to 7 weeks approximately
Weight at dosing: 218-220 g for the males; 172-174 g for the females
Source: [redacted], France
Acclimation period: 9 days
Diet: Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water: Tap water, *ad libitum*

Housing:	By sex in groups of 5 unless reduced by mortality or isolation, in suspended stainless steel wire mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C
	Humidity: 55 ± 15%
	Air changes: Approximately 15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates

13 February to 25 June, 2008 at [redacted], France.

2. Animal assignment and treatment

There were 10 animals of each sex per dose group. Animals were assigned to dose groups using a randomization by weight. BYI 02960 was administered in the diet for at least 90 days to Wistar rats at the following doses - 0, 100, 500 and 2500 ppm (equating approximately to 6.0, 30.2 and 156 mg/kg/day in males and 7.6, 38.3 and 186 mg/kg/day in females). A negative control group received plain diet. An additional, 10 males and 10 females fed either 0 or 2500 ppm of test diet for at least 90 days were maintained on control diet for a further 28 days to examine the reversibility of any effects seen. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Table 5.3.2-01: Study design

Test group	Concentration in diet (ppm)	Dose per animal (average)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	10 + 10 *	10 + 10 *
2	100	6.0	7.6	10	10
3	500	30.2	38.3	10	10
4	2500	156	186	10 + 10 *	10 + 10 *

* : These animals will be sacrificed following at least a 28-day recovery period after termination of treatment.

3. Diet preparation and analysis

The test substance was incorporated into the diet by dry mixing to provide the required dietary concentrations of 100, 500 or 2500 ppm. Initially, there were two preparations (F1 and F2) for the study. For the highest concentration of 2500 ppm, a second preparation (F2bis) was performed due to unacceptable results concerning the concentration of the second formulation of 2500 ppm.
The stability of the test substance in the diet (concentrations of 20, 100 and 2500 ppm) was checked over a period of at least 92 or 81 days of frozen storage followed by 10 days at room temperature or for at least 102 or 91 days of storage at room temperature. BYI 02960 formulation at 2500 ppm was found

to be stable in diet after at least 102 days of storage at room temperature and after 92 days of frozen storage followed by 10 days at room temperature.
Samples of the 20 and 100 ppm preparations were found to be stable in diet after 81 and 92 days, respectively, of frozen storage followed by 10 days at room temperature. They were also found to be stable after at least 21 days of storage at room temperature.
The homogeneity of BYI 02960 in diet was verified on the first preparation at the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean values obtained from the homogeneity check were taken as measured concentration. The dietary levels of the test substance were verified for each concentration on each preparation.

4. Statistics
Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods
1. Clinical signs and mortality
Animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily for all animals during the study. Out of cage detailed physical examinations were performed at least weekly during the treatment and recovery periods.

2. Neurological examination
During study Weeks 12 to 13, a neurotoxicity assessment was performed on all surviving animals from the main study groups by observers who were blind with respect to the dose level.

Exploratory locomotor activity
Animals were tested individually using an automated photocell recording apparatus (Imetronic, Bordeaux, France) designed to measure quantitatively spontaneous exploratory locomotor activity in a novel environment. Exploratory locomotor activity was recorded during the first 90 minutes with data being collected at regular intervals throughout the session.

Open field observations
Changes in gait, posture, as well as presence of clonic or tonic movements, stereotypic behavior (*e.g.* excessive grooming, repetitive circling), bizarre behavior (*e.g.* selfmutilation, walking backward) and other neurological-related changes were recorded for all surviving animals.

Sensory reactivity

The following reflexes and responses were recorded:

- Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary constriction by focusing a narrow beam of light in the eyes)
- Surface righting reflex (by putting the animal on its back and measuring its ability/rapidity to reassume a normal standing position)
- Corneal reflex (by touching the medial canthus with a fine object and observing the quick and complete close of the eyelids)
- Flexor reflex (by pinching the toes and measuring the presence/strength of the flexor response of each hindlimb)
- Auditory startle response (by measuring the animal response to an auditory stimulus)
- Tail pinch response (by pinching the tail with forceps and measuring the animal's reaction).

Grip strength

The fore- and hindlimb grip strength of all animals were measured quantitatively using a grip strength tester equipped with one pull or one push strain gauge (Bioseb, Chaville, France). The mean of three successive measurements was noted for both fore- and hindlimb grip strength.

3. Body weight

Each animal was weighed twice during the acclimatization period, on the first day of test substance administration, then at least weekly throughout the treatment and recovery periods. Additionally, diet fasted animals were weighed before scheduled necropsy.

4. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded twice weekly during the first 6 weeks of treatment, then weekly for all animals during the treatment and recovery periods. From these records the mean daily consumption was calculated. Food spillage was also noted.

The mean achieved dosage intake in mg/kg/day 13 was calculated for each sex for each week and for Weeks 1 to 13.

5. Ophthalmological examination

During the acclimatization period all animals were subjected to an ophthalmological examination. After instillation of an atropinic agent (Mydriaticum, Merck Sharp and Dohme) each eye was examined by means of an indirect ophthalmoscope. During Week 13 of the treatment period, all animals from control and high dose group were re-examined.

6. Test substance analysis

During Week 13 of the study, an additional blood sample was collected from the sublingual vein of the first five suitable animals of each group. Animals were not overnight dietary fasted before blood sampling. Plasma was prepared from blood collected into heparinised vials by centrifugation for further determination of the test substance and its major metabolites.

7. Hematology and clinical chemistry

On the day of scheduled sacrifices (Study Days 95, 96 or 97 for animals of the dosing phase and recovery Days 30 or 31 for the animals of the recovery phase), blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus prior to sacrifice. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane (Baxter, Maurepas, France). Blood was collected on EDTA for hematology, on lithium heparin (for plasma) and clot activator (for serum) for clinical chemistry and on sodium citrate for coagulation parameters.

The following haematology parameters were assayed using a Advia 120 (Bayer Diagnostics, [redacted], France): red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, reticuloeyte count, white blood cell count and differential count evaluation and platelet count. A blood smear was prepared and stained with Wright stain. It was examined when results of Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL Elite Pro (Instrumentation Laboratory, Paris, France). A blood smear was prepared and stained using May-Grünwald-Giemsa method. It was examined when the results of Advia 120 determinations were abnormal.

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus concentrations and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed on plasma samples, total protein and albumin concentrations were assayed on serum samples using an Advia 1650 (Siemens, Eragny, France). Globulin and albumin/globulin ratio values were calculated.

8. Urinalysis

On study Days 90 or 91 (for animals of the dosing phase) and on recovery Day 26 (for animals of the recovery phase), in the morning, overnight urine samples were collected from all animals in all groups. Food and water were not accessible during urine collection.

Urine samples were weighed to determine urinary volume and pH was assayed using a Clinitek 500 and Multistix dipsticks (Siemens, Eragny, France). Urinary refractive index was measured using a RFM320 refractometer (Bioblock Scientific, Illkirch, France).

Glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen were assayed using a Clinitek 500 and Multistix dipsticks (Siemens, Eragny, France).

Microscopic examination of the urinary sediment was performed after centrifugation of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was graded.

9. Sacrifice and pathology

On study Days 95, 96 or 97 of the dosing phase and on Days 30 or 31 of the recovery phase for the reversibility phase, all animals from corresponding groups were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day at the dosing phase and recovery sacrifice. Animals were diet fasted overnight prior to sacrifice.

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

Adrenal gland, brain, epididymides, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.
The following organs or tissues were sampled: adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, oesophagus, exorbital (lachrymal) gland, eye and optic nerve, Harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina. Three transverse sections of the brain were made for histopathological examination. The first section was done at the level of the optic chiasm including the basal ganglia, the septum, the cortex and the anterior hypothalamus. The second section was done at the level of hippocampus containing the cortex and the brain stem at the transition of diencephalon to mesencephalon. The third section contained the cerebellum and the brain stem (medulla oblongata).
A bone marrow smear was prepared from femur, stained with May-Grünwald Giemsa, but not examined, since no Advia 120 determinations were abnormal. Tissues samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative. Histopathological examinations were performed on all tissues from all the animals in the control and high dose groups and all decedents in all groups. The liver, kidney, lung, spleen, thyroid gland, thymus and urinary bladder were examined in all animals in the study.

II. Results and discussion

A. Observations

1. Clinical signs of toxicity

No treatment-related clinical signs were noted at any dose level in either sex. The few clinical signs observed were considered not to be related to BYI 02960 administration, as they were evenly distributed across the groups including the controls with no evidence of a dose-related effect.

2. Mortality

There were no mortalities during the study.

3. Neurological examinations

Exploratory locomotor activity

No treatment-related changes were recorded in overall mean exploratory locomotor activity at any dose level in either sex. In addition, the pattern of the locomotor activity over time in treated groups was similar to the controls.

Open field observations

No treatment-related changes were recorded during the open field observation at any dose level in either sex. The few changes noted in both sexes were considered to reflect inter individual variations and/or were observed with no dose-relationship and were thus considered not to be treatment-related.

Sensory reactivity

All reflexes and responses evaluated were unaffected by the treatment at any dose level in either sex. The few changes noted in both sexes were considered to reflect inter-individual variations and/or were observed with no dose-relationship and were thus considered not to be treatment-related.

Grip strength

No treatment-related changes were recorded at the fore- and hindlimb grip strength measurements at any dose level in either sex.

B. Body weight and body weight gain

1. Dosing phase

At 2500 ppm, a lower body weight was observed in both sexes throughout the study. A reduced mean body weight gain/day was observed in males at several occasions during the first two months of the study, whereas it was only noted during the first week of the treatment for female rats. At the end of the treatment period (Study Day 92), the mean body weight was 6% lower than the controls for males and females. At the end of the treatment period, the overall mean body weight gain was 12% and 15% lower than the controls in males and females, respectively.

At 500 ppm, a reduced mean body weight gain/day was observed in females during the first week and during the last week of the study. The overall mean body weight gain was 12% lower than the controls in females, at the end of the treatment period (Study Day 92).

At 100 ppm, body weight and body weight gain were comparable to the control values.

Table 5.3.2-02: Group mean body weight (BW) and cumulative body weight gain (BWG) (g)

BYI 02960 Dose levels (ppm)	0	100	500	2500
Male				
Initial BW (Day 1) (%C)	220	220 (100)	218 (99)	219 (100)
BW Week 1 (Day 8) (%C)	280	280 (100)	274 (98)	260 (93)**
BW Week 4 (Day 29) (%C)	393	400 (102)	389 (99)	368 (94)**
BW Week 8 (Day 57) (%C)	470	485 (103)	463 (99)	436 (93)**
Final BW Week 13 (Day 92) (%C)	**512**	**528 (103)**	**505 (99)**	**479 (94)**
BWG Weeks 1-4 (Days 1 to 29) (%C)	174	180 (103)	171 (98)	149 (86)**
BWG Weeks 1-8 (Days 1 to 57) (%C)	251	266 (106)	245 (98)	216 (86)**
Overall BWG (Days 1 to 92) (%C)	**293**	**308 (105)**	**287 (98)**	**259 (88)***
Female				
Initial BW (Day 1) (%C)	**174**	**174 (100)**	**174 (100)**	**172 (99)**
BW Week 1 (Day 8) (%C)	199	200 (101)	194 (97)	180 (90)**
BW Week 4 (Day 29) (%C)	244	249 (102)	238 (98)	229 (94)**
BW Week 8 (Day 57) (%C)	271	277 (102)	260 (96)*	254 (94)**
Final BW Week 13 (Day 92) (%C)	**284**	**284 (100)**	**271 (95)**	**266 (94)****
BWG Weeks 1-4 (Days 1 to 29) (%C)	70	75 (107)	64 (91)	56 (80)**
BWG Weeks 1-8 (Days 1 to 57) (%C)	97	102 (105)	86 (89)*	81 (84)**
Overall BWG (Days 1 to 92) (%C)	**110**	**110 (100)**	**97 (88)***	**94 (85)****

C : control
** : Statistically different ($p < 0.01$) from the control
* : Statistically different ($p < 0.05$) from the control

2. Recovery

Throughout the recovery phase of the study the mean body weight of males and females remained lower than that of the control group. The body weight gain of both males and females was higher than controls by 220 and 160% during the first week of the recovery phase. Thereafter, the mean body weight gain was comparable to or higher than the controls throughout the recovery phase of the study.

Table 5.3.2-03: Group mean body weight (BW) and cumulative body weight gain (BWG) (g)

	Male		Female	
BYI 02960 Dose levels (ppm)	0	2500	0	2500
Initial BW (Recovery Day 1) (%C)	511	476(93)	290	272 (94)**
Final BW (Recovery Day 29) (%C)	527	514 (98)	297	290 (98)
Overall BWG (Recovery Days 1-29) (%C)	16	38 (238)**	7	17 (243)*

C : control
** : Statistically different ($p < 0.01$) from the control
* : Statistically different ($p < 0.05$) from the control.

C. Food consumption and compound intake

1. Dosing phase

At 2500 ppm, a slight reduction in mean food consumption (17%) was observed for males during the first four days of the study and thereafter on several occasions (approximately 5%) from Study Day 71 until the end of the dosing phase. In females, a reduction in mean food consumption was observed from the first week of the study until Study Week 7 (between 9% and 29%. Thereafter, there was no evidence of a treatment-related effect on food consumption.
No treatment-related effects on mean food consumption were observed for both sexes at dose levels of 500 and 100 ppm compared to the controls.

Table 5.3.2-04: Mean food consumption per day (FC) (g)

BYI 02960 Dose levels (ppm)	**0**	**100**	**500**	**2500**
Male				
Initial FC (Day 4) (%C)	**24.1**	**23.4 (97)**	**23.4 (97)**	**20.1 (83)****
FC Week 1 (Day 8) (%C)	25.4	25.0 (98)	24.1 (95)	24.8 (98)
FC Week 4 (Day 29) (%C)	28.9	28.7 (99)	25.3 (98)	25.5 (98)
FC Week 8 (Day 57) (%C)	25.5	26.4 (104)	25.8 (101)	24.5 (96)
Final FC Week 13 (Day 92) (%C)	**23.8**	**22.5 (95)**	**22.0 (92)**	**22.1 (93)**
Female				
Initial FC (Day 4) (%C)	**18.2**	**17.9 (98)**	**17.0 (93)**	**13.0 (71)****
FC Week 1 (Day 8) (%C)	18.9	19.5 (103)	17.2 (91)	16.20(86)*
FC Week 4 (Day 29) (%C)	19.8	19.4 (98)	19.0 (96)	16.9 (85)**
FC Week 8 (Day 57) (%C)	19.1	19.9 (104)	18.4 (96)	18.0 (94)
Final FC Week 13 (Day 92) (%C)	**17.8**	**15.8 (89)**	**16.3 (92)**	**16.0 (90)**

C : control
** : Statistically different ($p < 0.01$) from the control
* : Statistically different ($p < 0.05$) from the control.

The mean achieved dosage intake per group was: 6.0, 30 and 156 mg/kg/day in males and 7.6, 38 and 186 mg/kg/day in females.

2. Recovery
No treatment-related effect on mean food consumption was noted during the recovery phase in either sex.

D. Ophthalmoscopic examination

There were no treatment-related ophthalmological abnormalities at ophthalmological examination following 91 days of exposure. Hemorrhage of the iris and a damaged eye were noted in one male (ST4M0480) and one female (ST4F0499), respectively, exposed to 2500 ppm. In view of the single occurrence, these findings were considered incidental.

E. Blood analysis

1. Haematological findings
Dosing phase

At 2500 ppm, for females, mean platelet count was slightly higher when compared to the control group (+15%, p <0.05). No treatment-related change was observed in females administered lower doses or in males at any dietary level.

Recover phase

The slight changes observed at the end of the dosing phase were considered to be reversible as no relevant change was noted after the recovery period.

2. Clinical chemistry findings

Dosing phase

Treatment-related changes were noted at 2500 ppm in both sexes. Mean total bilirubin and glucose concentrations were slightly lower whereas mean total cholesterol and triglycerides concentrations were slightly higher when compared to the control groups. No relevant change was observed at 500 and 100 ppm in either sex.

Table 5.3.2-05: Significant clinical chemistry changes
(Mean ± Standard deviation; % change when compared to controls)

BYI 02960 Dose levels (ppm)	0	100	500	2500
Male				
Total Bilirubin (µmol/l)	1.3 ± 0.3	1.1 ± 0.3 (- 15%)	1.1 ± 0.2 (- 15%)	0.8 ± 0.2** (- 38%)
Glucose (mmol/l)	6.59 ± 1.10	6.56 ± 0.58 (0%)	6.19 ± 0.91 (- 6%)	5.23 ± 0.40** (- 21%)
Total cholesterol (mmol/l)	1.54 ± 0.33	1.78 ± 0.23 (+ 16%)	1.70 ± 0.16 (+ 10%)	1.97 ± 0.44 (+ 28%)
Triglycerides (mmol/l)	0.85 ± 0.32	1.03 ± 0.36 (+ 21%)	0.85 ± 0.39 (0%)	1.15 ± 0.48 (+ 35%)
Female				
Total Bilirubin (µmol/l)	2.0 ± 0.5	1.91 ± 0.5 (- 5%)	1.8 ± 0.4 (- 10%)	1.1 ± 0.3** (- 45%)
Glucose (mmol/l)	6.03 ± 0.78	5.82 ± 0.97 (- 3%)	5.58 ± 0.46 (- 7%)	4.69 ± 0.54** (- 22%)
Total cholesterol (mmol/l)	1.55 ± 0.24	1.68 ± 0.35 (+ 8%)	1.86 ± 0.37 (+ 20%)	2.26 ± 0.28** (+ 46%)
Triglycerides (mmol/l)	0.44 ± 0.12	0.44 ± 0.26 (0%)	0.48 ± 0.13 (+ 9%)	0.73 ± 0.32 (+ 66%)

** : Statistically different (p <0.01) from the control

Recover phase

Total bilirubin variation was considered to be partially reversible in females at the end of the recovery phase as the mean concentration remained slightly lower (- 25%, p <0.05, relative to the control group). The other treatment-related changes observed after BYI 02960 administration were considered to be reversible as no relevant differences were noted after the recovery period. The other statistically significant differences were considered not to be relevant in view of the variation of the individual values.

F. Urinalysis

1. Dosing phase
No treatment-related change was noted at any dose level in either sex.

2. Recovery phase
No relevant change was noted.

G. Sacrifice and pathology

1. Organ weight
Dosing phase
Treatment-related changes were noted in the terminal body weight and the weights of liver and thyroid gland.
At 2500 ppm, a lower mean terminal body weight was observed in females (- 6%, $p < 0.05$) and in males (- 7%, not statistically significant) when compared to controls. At 2500 ppm, mean liver to body weight ratio was statistically significantly higher in both sexes when compared to controls. At 2500 ppm, mean thyroid gland to body weight ratio was statistically significantly higher in males when compared to controls. These changes, even if partly due to lower terminal body weight, were associated with relevant histopathological findings and were considered to be treatment-related. At 500 ppm, mean thyroid gland to body weight ratio was statistically significantly higher in males when compared to controls. Since this change was not associated with any relevant microscopic finding, it was considered not to be toxicologically relevant. The few other organ weight differences were considered to be incidental and not treatment-related.

Table 5.3.2-06: Significant organ weight changes (Mean ± Standard deviation; % change when compared to controls)

Sex	Male				Female			
Dose level (ppm)	**0**	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
Liver								
Absolute weight (g)	10.92 ± 1.44	11.37 ± 1.85 (+ 4%)	10.92 ± 1.01 (0%)	11.78 ± 1.43 (+ 8%)	6.18 ± 0.27	5.91 ± 0.58 (- 4%)	5.93 ± 0.29 (- 4%)	6.65 ± 0.82 (+ 8%)
Organ weight to body weight ratio (%)	2.194 ± 0.164	2.231 ± 0.172 (+ 2%)	2.255 ± 0.155 (+ 3%)	2.546** ± 0.200 (+ 16%)	2.290 ± 0.115	2.170 ± 0.095 (- 5%)	2.283 ± 0.086 (0%)	2.624** ± 0.232 (+ 15%)
Organ weight to brain weight ratio (%)	504.824 ± 63.640	527.855 ± 86.107 (+ 5%)	494.455 ± 62.605 (- 2%)	546.074 ± 65.306 (+ 8%)	303.728 ± 16.528	290.220 ± 29.694 (- 4%)	294.529 ± 11.795 (- 3%)	333.353 ± 42.388 (+ 10%)
Thyroid gland								
Absolute weight (g)	0.0195 ± 0.0028	0.0222 ± 0.0031 (+ 14%)	0.0228 ± 0.0027 (+ 17%)	0.0234 ± 0.0046 (+ 20%)	0.0162 ± 0.0023	0.0162 ± 0.0017 (0%)	0.0165 ± 0.0026 (+ 2%)	0.0171 ± 0.0028 (+ 6%)
Organ weight to body weight ratio (%)	0.00393 ± 0.00042	0.00439 ± 0.00055 (+ 12%)	0.00473* ± 0.00068 (+ 20%)	0.00494** ± 0.00078 (+ 26%)	0.00602 ± 0.00095	0.00599 ± 0.00095 (0%)	0.00633 ± 0.00095 (+ 5%)	0.00679 ± 0.00108 (+ 13%)
Organ weight to brain weight ratio (%)	0.90330 ± 0.12280	1.02835 ± 0.12300 (+ 14%)	1.02950 ± 0.11047 (+ 14%)	1.08348 ± 0.21085 (+ 20%)	0.79926 ± 0.12971	0.79390 ± 0.09540 (- 1%)	0.81569 ± 0.11771 (+ 2%)	0.85994 ± 0.14222 (+ 8%)

** :p <0.01 * :p <0.05

Recovery phase

At the end of the recovery phase, no differences were observed in terminal body weight, liver weights and thyroid gland weights. All the treatment-related changes observed at the end the treatment period were thus considered to be reversible.

2. Gross and histopathology

Dosing phase

Treatment-related macroscopic changes were noted in the liver and thyroid gland.

At 2500 ppm, enlarged liver was observed in four males and one female. This finding was correlated to microscopic hepatocellular hypertrophy and was considered to be treatment-related.

At 2500 ppm, dark thyroid gland was observed in 1/10 males. This finding was correlated to microscopic follicular cell hypertrophy and was considered to be treatment-related. All other macroscopic changes were considered as incidental and not treatment-related.

Treatment-related microscopic findings were observed in the liver and thyroid gland.
At 2500 ppm in the liver, minimal to slight centrilobular hepatocellular hypertrophy was observed in both sexes.

Table 5.3.2-07: Incidence of selected pathological findings

Sex	**Male**				**Female**			
BYI 02960 Dose level (ppm)	0	100	500	2500	0	100	500	2500
Number of animals examined	10	10	10	10	10	10	10	10
Liver: Centrilobular hypertrophy: diffuse								
Minimal	0	0	0	6	0	0	0	3
Slight	0	0	0	4	0	0	0	0
Total	**0**	**0**	**0**	**10**	**0**	**0**	**0**	**3**
Thyroid gland: Follicular cell hypertrophy: diffuse								
Minimal	0	0	0	3	0	0	0	0
Total	**0**	**0**	**0**	**3**	**0**	**0**	**0**	**0**

Recovery phase

At the end of the recovery phase, no treatment-related macroscopic findings were observed in the liver and the thyroid gland. All the treatment-related findings observed at the end of the treatment period were thus considered to be reversible.
At 2500 ppm in the thyroid gland, minimal follicular cell hypertrophy was observed in males. All other microscopic changes were considered as incidental and not treatment-related.
At the end of the recovery phase, no treatment-related findings were observed in the liver and the thyroid gland. All the treatment-related findings observed at the end of the treatment period were thus considered to be reversible.

III. Conclusions

In conclusion, a dose level of 500 ppm of BYI 02960 represented a No Observed Adverse Effect Level (NOAEL) for both sexes (approximately 30.2 mg/kg/day for males and 38.3 mg/kg/day for females).

Oral 90-day toxicity in the mouse

Report:	**KIIA 5.3.2/02 [redacted] M.; 2009**
Title:	BYI 02960, 90-day toxicity study in the mouse by dietary administration
Report No & Document No	SA 07295; M-328668-03-2
Guidelines:	OECD 408 (1998); EEC Directive 2001/59/EC, Method B.26 (August, 2001),EPA Health Effects Test Guideline (OPPTS 870.3100; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
GLP	Yes (certified laboratory)

Executive Summary

BYI 02960 (batch number NLL 7780-44-6: a pink powder, 99.5% w/w purity) was administered continuously via dietary admixture. Groups of C57BL/6J mice (10/sex/group) received the test substance at concentrations of 100, 500 and 2500 ppm (equating approximately to 16, 81, 407 mg/kg body weight/day in males and 19, 98, 473 mg/kg body weight/day in females, respectively) for at least 90 days. A similarly constituted group of 10 males and 10 females received untreated diet and acted as a control group.

BYI 02960 dietary administration to male and female C57BL/6J mice at 100, 500 and 2500 ppm for at least 90 days induced no treatment-related mortalities and no clinical signs.

At 2500 ppm

A lower body weight was observed in both sexes throughout the study. Mean body weight gain/day was reduced between Study Days 1 to 22 in males and between Study Days 1 and 8 in females. Thereafter, mean body weight gain per day for both sexes was similar to the control groups. The overall cumulative mean body weight over the entire study duration (Days 1 to 92) was lower by 43% in males and by 7% in females.

A slight reduction of 10 and 11% in mean food consumption was observed for females between Study Days 1 and 22 when compared to controls. Clinical chemistry evaluation revealed a lower mean total cholesterol concentration in both sexes (- 30% and -24% in males and females, respectively) as compared to the controls. Higher mean alkaline phosphatase activity was noted in males (+ 38%) whereas mean alanine and aspartate aminotransferase activities were higher in females (+ 106% and + 36%, respectively). In both sexes, mean urea concentrations were higher (+ 51% and + 19% in males and females, respectively) and total protein concentrations were slightly lower (- 5%). In females, mean albumin concentrations were slightly lower (-8%). Mean terminal body weight was statistically significantly lower in males (- 11 %) when compared to controls. Mean absolute and relative liver weights were statistically significantly higher in females (+ 12% to 18%) when compared to controls. Mean absolute kidney weight and mean kidney to brain weight ratio were statistically significantly lower (- 11%) in males when compared to controls. At macroscopic examination pale liver was noted in 6/10 females. At microscopic examination, a slight increase in severity of diffuse hepatocellular vacuolation was noted in the liver in both sexes. In the kidney, a loss of the normal multifocal/diffuse cortical epithelial vacuolation was noted in males.

At 500 ppm
Mean body weight gain/day in males was 0.09 g/day compared to 0.20 g/day in the controls between Study Days 1 and 8 only. However, this slight body weight gain decrease was not considered adverse.

At 100 ppm
There were no treatment-related changes in any parameters assessed.

In conclusion, the dose of 500 ppm of BYI 02960 was considered to be the No Observed Adverse Effect Level (NOAEL), equating to 80.6 and 98.1 mg/kg body weight/day in males and females, respectively.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Pink powder
Lot/Batch:	NLI-7780-44-6
Purity:	99.5%
CAS:	951659-40-8
Stability of test compound:	Stable at 20 and 100 ppm when stored frozen for 81 or 92 days followed by 10 days at ambient temperature or after 21 at ambient temperature; stable at 2500 ppm when stored frozen for 92 days followed by 10 days at ambient temperature or after 102 days at ambient temperature
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Mouse
Strain:	C57BL/6J
Age:	6 weeks approximately
Weight at dosing:	20.0 to 20.4 g for the males; 16.2 to 16.8 g for the females
Source:	[redacted] France
Acclimation period:	12 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	By sex in groups of 3 from arrival to pre-study day 5, individually thereafter, in suspended stainless steel wire mesh cages

Environmental conditions: Temperature: 22 ± 2 °C
Humidity: 55 ± 15%
Air changes: Approximately 10 to 15 changes per hour
Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design and methods

1. In life dates

05 March to 19 June, 2008 at [redacted] France.

2. Animal assignment and treatment

There were 10 animals of each sex per dose group. Animals were assigned to dose groups using a randomization by weight. BYI 02960 was administered in the diet for at least 90 days to C57BL/6J mice at the following doses - 0, 100, 500 and 2500 ppm (equating approximately to 16, 81, 407 mg/kg body weight/day in males and 19, 98, 473 mg/kg body weight/day in females, respectively). A negative control group received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Table 5.3.2-08: Study design

Test group	Concentration in diet (ppm)	Dose per animal (average)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	10	10
2	100	16	19	10	10
3	500	81	98	10	10
4	2500	407	473	10	10

3. Diet preparation and analysis

The test substance was incorporated into the diet by dry mixing to provide the required dietary concentrations of 100, 500 or 2500 ppm. Initially, there were two preparations (Fl and F2) for the study The stability of the test substance in the diet (concentrations of 20, 100 and 2500 ppm) was checked over a period of at least 92 or 81 days of frozen storage followed by 10 days at room temperature or for at least 102 or 91 days of storage at room temperature. BYI 02960 formulation at 2500 ppm was found to be stable in diet after at least 102 days of storage at room temperature and after 92 days of frozen storage followed by 10 days at room temperature.

Samples of the 20 and 100 ppm preparations were found to be stable in diet after 81 and 92 days, respectively, of frozen storage followed by 10 days at room temperature. They were also found to be stable after at least 21 days of storage at room temperature.

The homogeneity of BYI 02960 in diet was verified on the first preparation at the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean values obtained from the

homogeneity check were taken as measured concentration. The dietary levels of the test substance were verified for each concentration on each preparation.

4. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods

1. Clinical signs and mortality

Animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily for all animals during the study. Out of cage detailed physical examinations were performed at least weekly during the treatment.

2. Body weight

Each animal was weighed twice during the acclimatization period, on the first day of test substance administration, then at least weekly throughout the treatment and recovery periods. Additionally, diet fasted animals were weighed before scheduled necropsy.

3. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment. From these records the mean daily consumption was calculated. Food spillage was also noted.
The mean achieved dosage intake in mg/kg/day was calculated for each sex for each week and for Weeks 1 to 13.

4. Clinical chemistry

On the day of scheduled sacrifices (Study Days 93, 94 or 95), blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus prior to sacrifice. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane (Baxter, Maurepas, France). Blood was collected on lithium heparin for plasma clinical chemistry.
Any significant change in the general appearance of the plasma was recorded. Total bilirubin, urea, creatinine, total cholesterol concentrations and aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed on plasma samples, using an Advia 1650 (Siemens, Eragny, France).

5. Test substance analysis

During Week 12 of the study, an additional blood sample was collected from the retro-orbital venous plexus of the first five suitable animals of each group. Animals were not overnight dietary fasted before blood sampling. Prior to blood sampling, animals were anesthetized with isoflurane (Baxter, Maurepas,

France). Plasma was prepared from blood collected into heparinised vials by centrifugation for further determination of the test substance and its major metabolites. Samples were stored in the dark at approximately -20 °C until possible analysis.

6. Sacrifice and pathology

On study Days 93, 94 or 95, all surviving animals were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Significant macroscopic abnormalities were recorded, sampled and examined microscopically.

Adrenal gland, brain, heart, kidney, liver, spleen, testis, thymus, and uterus (including cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.
The following organs or tissues were sampled: adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, oesophagus, exorbital (lachrymal) gland, eye and optic nerve, Harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pharynx, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina. Three transverse sections of the brain were made for histopathological examination. The first section was done at the level of the optic chiasm including the basal ganglia, the septum, the cortex and the anterior hypothalamus. The second section was done at the level of hippocampus containing the cortex and the brain stem at the transition of diencephalon to mesencephalon. The third section contained the cerebellum and the brain stem (medulla oblongata).

A bone marrow smear was prepared from femur, stained with May-Grünwald Giemsa, but not examined as any treatment-related changes were observed in bone marrow histology. Tissues samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative. Histopathological examinations were performed on all tissues (except exorbital lachrymal gland, larynx/pharynx and nasal cavities that were not processed and kept in fixative for possible future examination) from all the animals in the control and high dose groups and all decedents in all groups. The liver, kidney, lung, thyroid gland and urinary bladder were examined in all animals in the study.

II. Results and discussion

A. Observations

1. Clinical signs

There were no treatment-related clinical signs observed during the study in either sex of all groups. The few clinical signs observed were considered not to be related to BYI 02960 administration, as they were evenly distributed across the groups, including the controls, with no dose-related increase.

2. Mortality

There were no treatment-related mortalities during the study. One female from the 500 ppm group was killed for humane reasons on Study Day 61. Macroscopic examination revealed dilated and tear in esophagus, food content in thoracic cavity, enlarged adrenal glands, dark liver, white foci on stomach, atrophic/small thymus and enlarged bronchial lymph node. The cause of death of this animal was due to an initial esophageal impaction followed by tearing and a subsequent locally extensive inflammatory reaction and was considered as incidental.

B. Body weight and body weight gain

At 2500 ppm, a lower body weight was observed in both sexes throughout the study ($p < 0.05$ or $p < 0.01$). Mean body weight gain/day was reduced between Study Days 1 to 22 in males and between Study Days 1 and 8 in females. Thereafter, mean body weight gain per day for both sexes was similar to the control groups. The overall cumulative mean body weight over the entire study duration (Days 1 to 92) was lower by 43% ($p < 0.01$) in males and by 7% in females (not statistically significant).
At 500 ppm, mean body weight gain/day in males was 0.09 g/day ($p < 0.01$) compared to 0.20 g/day in the controls, between Study Days 1 and 8 only. Thereafter, mean body weight gain per day was comparable to the control group.
At 100 ppm, the lower mean body weight gain of + 0.11 g/day ($p < 0.05$) observed in males between Study Days 1 and 8 only, compared with a gain of + 0.20 g/day in the corresponding controls, was considered to be incidental.

Table 5.3.2-09: Group mean body weight (BW) and cumulative body weight gain (BWG) (g)

BYI 02960 Dose level (ppm)	0	100	500	2500
Male				
Initial BW (Day 1) (%C)	20.0	20.2 (101)	20.3 (102)	20.4 (102)
BW Week 1 (Day 8) (%C)	21.4	21.0 (98)	20.9 (98)	19.8 (93)**
BW Week 4 (Day 29) (%C)	23.5	23.5 (100)	23.0 (98)	21.0 (89)**
BW Week 8 (Day 57) (%C)	25.7	25.8 (100)	25.2 (98)	23.2 (90)**
Final BW Week 13 (Day 92) (%C)	**27.2**	**27.1 (100)**	**26.8 (99)**	**24.4 (90)****
BWG Weeks 1-4 (Days 1 to 29) (%C)	3.4	3.3 (97)	2.7 (79)	0.6 (18)**
BWG Weeks 1-8 (Days 1 to 57) (%C)	5.7	5.5 (96)	4.9 (86)	2.8 (49)**
Overall BWG (Days 1 to 92) (%C)	**7.2**	**6.9 (96)**	**6.5 (90)**	**4.1 (57)****
Female				
Initial BW (Day 1) (%C)	16.8	16.6 (99)	16.7 (99)	16.2 (96)
BW Week 1 (Day 8) (%C)	18.0	17.6 (98)	17.4 (97)	16.8 (93)**
BW Week 4 (Day 29) (%C)	20.2	19.8 (98)	19.6 (97)	18.7 (93)**
BW Week 8 (Day 57) (%C)	21.4	21.4 (100)	20.8 (97)	20.1 (94)**
Final BW Week 13 (Day 92) (%C)	**22.2**	**22.2 (100)**	**21.8 (98)**	**21.2 (95)***
BWG Weeks 1-4 (Days 1 to 29) (%C)	3.4	3.3 (97)	2.9 (85)	2.5 (74)
BWG Weeks 1-8 (Days 1 to 57) (%C)	4.6	4.8 (104)	4.1 (89)	3.9 (85)
Overall BWG (Days 1 to 92) (%C)	**5.4**	**5.7 (106)**	**5.2 (96)**	**5.0 (93)**

C : control
** : Statistically different ($p < 0.01$) from the control
* : Statistically different ($p < 0.05$) from the control.

C. Food consumption and compound intake

At 2500 ppm, a slight reduction of 10 to 11% in mean food consumption was observed for females between Study Days 1 and 22, when compared to controls. In males, there was no evidence of a treatment-related effect on food consumption.
No treatment-related effects on mean food consumption were observed for both sexes at dose levels of 500 and 100 ppm compared to the controls.

Table 5.3.2-10: Mean food consumption per day (FC) (g)

BYI 02960 Dose level (ppm)	0	100	500	2500
Male				
Initial FC (Day 8) (%C)	**3.8**	**3.9 (103)**	**3.8 (100)**	**3.6 (95)**
FC Week 4 (Day 29) (%C)	3.7	3.8 (103)	4.0 (108)*	3.6 (97)
FC Week 8 (Day 57) (%C)	3.7	3.9 (105)	4.0 (108)	3.6 (97)
Final FC Week 13 (Day 92) (%C)	**3.9**	**4.0 (103)**	**4.0 (103)**	**3.7 (95)**
Female				
Initial FC (Day 8) (%C)	**3.6**	**3.6 (100)**	**3.5 (97)**	**3.2 (89)***
FC Week 4 (Day 29) (%C)	3.9	3.7 (95)	3.9 (100)	3.6 (92)
FC Week 8 (Day 57) (%C)	4.1	4.0 (98)	4.0 (98)	3.8 (93)
Final FC Week 13 (Day 92) (%C)	**4.1**	**4.0 (98)**	**4.3 (105)**	**4.0 (98)**

C : control
* : Statistically different ($p < 0.05$) from the control.

The mean dosage intake achieved per group was: 15.6, 80.6 and 407 mg/kg/day in males and 18.8, 98.1 and 473 mg/kg/day in females.

D. Blood analysis

1. Clinical chemistry

Treatment-related changes were observed at 2500 ppm when compared to the controls.
Mean total cholesterol concentrations were lower in both sexes. Higher mean alkaline phosphatase activity was noted in males whereas alanine and aspartate aminotransferase activities were higher in females. In both sexes, mean urea concentrations were higher, whereas total protein concentrations were slightly lower. In females, mean albumin concentrations were slightly lower.

No relevant change was observed at 500 and 100 ppm in either sex.

Table 5.3.2-11: Significant clinical chemistry variations

Clinical Chemestry Variations Mean ± standard deviation (% change when compared to controls)				
BYI 02960 Dose level (ppm)	**Control**	**2500**	**Control**	**2500**
Sex	**Male**		**Female**	
Total cholesterol (mmol/l)	1.91 ± 0.18 -	1.34 ± 0.14 ** (-30%)	1.52 ± 0.16 -	1.16 ± 0.17 ** (-24%)
Alkaline phosphatase (IU/l)	76 ± 11 -	105 ± 11 ** (+38%)	131 ± 14 -	144 ± 15 (+10%)
Alanine aminotransferase (IU/l)	27 ± 5 -	35 ± 9 (+30%)	36 ± 10 -	74 ± 38 * (+106%)
Aspartate aminotransferase (IU/l)	88 ± 15 -	105 ± 20 (+19%)	130 ± 21 -	177 ± 68 (+36%)
Urea (mmol/l)	11.14 ± 1.23 -	16.78 ± 2.85 ** (+51%)	12.76 ± 1.22 -	15.23 ± 2.46 * (+19%)
Total protein (g/L)	59 ± 2 -	56 ± 4 * (-5%)	58 ± 2 -	55 ± 2 ** (-5%)
Albumin (g/L)	34 ± 0 -	33 ± 2 (-3%)	36 ± 1 -	33 ± 1 ** (-8%)

** : Statistically different ($p < 0.01$) from the control
* : Statistically different ($p < 0.05$) from the control.

E. Sacrifice and pathology

1. Organ weight

Treatment-related changes were noted in terminal body weights, liver and kidney weights.

At 2500 ppm, mean terminal body weight was statistically significantly lower in males (- 11 %, $p \leq 0.01$) when compared to controls. Mean liver to body weight ratio was statistically significantly higher in males when compared to controls. This latter change was mainly attributed to the lower terminal body weights.

At 2500 ppm, mean absolute and relative liver weights were statistically significantly higher in females when compared to controls. At 2500 ppm, mean absolute kidney weight and mean kidney to brain weight ratio were statistically significantly lower in males when compared to controls.

Table 5.3.2-12: Significant organ weight changes

Sex	Male				Female			
BYI 02960 Dose level (ppm)	**0**	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
Liver								
Absolute liver weight (g)	0.96 ± 0.06	1.02 ± 0.08 (+ 6%)	0.95 ± 0.07 (- 1%)	1.02 ± 0.06 (+ 6%)	0.82 ± 0.04	0.82 ± 0.06 (0%)	0.84 ± 0.08 (+ 2%)	0.92** ± 0.07 (+ 12%)
Liver to body weight ratio (%)	4.170 ± 0.212	4.422 ± 0.264 (+ 6%)	4.207 ± 0.149 (+ 1%)	4.989** ± 0.362 (+ 20%)	4.474 ± 0.209	4.498 ± 0.186 (+ 1%)	4.609 ± 0.453 (+ 3%)	5.264** ± 0.437 (+ 18%)
Liver to brain weight ratio (%)	214.942 ± 12.390	223.158 ± 18.445 (+ 4%)	215.174 ± 14.193 (0%)	229.924* ± 17.249 (+ 7%)	181.519 ± 10.591	179.340 ± 11.537 (- 1%)	191.002 ± 18.699 (+ 5%)	205.623** ± 16.395 (+ 13%)
Kidney								
Absolute kidney weight (g)	0.35 ± 0.03	0.35 ± 0.03 (0%)	0.32 ± 0.03 (- 9%)	0.31** ± 0.02 (-11%)	0.26 ± 0.01	0.27 ± 0.02 (+ 4%)	0.26 ± 0.01 (0%)	0.25 ± 0.01 (- 4%)
Kidney to body weight ratio (%)	1.514 ± 0.115	1.531 ± 0.098 (+ 1%)	1.437 ± 0.097 (- 5%)	1.515 ± 0.092 (0%)	1.439 ± 0.060	1.474 ± 0.127 (+ 2%)	1.417 ± 0.050 (- 2%)	1.425 ± 0.045 (- 1%)
Kidney to brain weight ratio (%)	78.013 ± 6.005	77.257 ± 6.434 (- 1%)	73.414 ± 5.083 (- 6%)	69.793** ± 4.180 (-11%)	58.320 ± 2.117	58.675 ± 4.816 (+ 1%)	58.716 ± 3.072 (+ 1%)	55.709 ± 2.477 (- 4%)

2. Gross and histopathology

At macroscopic examination, pale liver was observed in 6/10 females administered at 2500 ppm. Other changes were considered as incidental and not treatment-related.

Treatment-related microscopic changes were noted in the liver and the kidney.

At 2500 ppm, in the liver, a slight increase in severity of diffuse hepatocellular vacuolation was noted in both sexes and Treatment-related changes were noted in the liver and the kidney.

At 2500 ppm, in the liver, a slight increase in severity of diffuse hepatocellular vacuolation was noted in both sexes and , a loss of the normal cortical epithelial vacuolation was noted in the kidney of males only.

Table 5.3.2-13: Significant microscopic changes

Sex	Male				Female			
BYI 02960 Dose level (ppm)	0	100	500	2500	0	100	500	2500
Number of animals examined	10	10	10	10	10	10	9	10
Liver: Hepatocellular vacuolation : diffuse								
Minimal	6	3	8	0	7	7	6	2
Slight	4	7	2	6	3	3	3	3
Moderate	0	0	0	4	0	0	0	5
Total	**10**	**10**	**10**	**10**	**10**	**10**	**9**	**10**
Kidney: Corticoepithelial vacuolation : multifocal/diffuse								
Minimal	7	4	5	0	0	0	0	0
Slight	3	2	3	0	0	0	0	0
Moderate	0	2	0	0	0	0	0	0
Total	**10**	**8**	**8**	**0**	**0**	**0**	**0**	**0**

III. Conclusions

In conclusion, the dose of 500 ppm of BYI 02960 was considered to be the No Observed Adverse Effect Level (NOAEL), equating to 80.6 and 98.1 mg/kg body weight/day in males and females, respectively.

KIIA 5.3.3 - Oral 90-day toxicity (dog)

Report:	**KIIA 5.3.3/01, [redacted] D.A.; 2010**
Title:	A 90-Day Toxicity Feeding Study in the Beagle Dog with Technical Grade BYI 02960
Report No & Document No	09-S76-QQ M-369978-01-2
Guidelines:	OECD 409 (1998); EPA Health Effects Test Guideline (OPPTS 870.3150; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
GLP	Yes (certified laboratory)

Executive Summary

BYI 02960 (batch number 2009-000239: a beige powder, 96.2% purity) was administered via the diet to beagle dogs (4/sex/dose) of both sexes at nominal concentrations of 0, 400, 1200 or 3600/2400 ppm for at least 90 days (equating to approximately 12, 33 or 102/85 mg/kg body weight/day in males and 12, 41 or 107/78 mg/kg body weight/day in females). The 3600 ppm dose group was reduced to 2400 ppm beginning Study Week 9 due to clinical signs seen in two of the dogs on Day 44 and continual weight loss in the high-dose group. The objective of lowering the dose was to prevent severe clinical signs of toxicity and reverse the weight loss.

Cage side observations and food consumption were recorded daily and detailed clinical observations and body weights were recorded weekly. Hematology, clinical chemistry, and urinalysis evaluations were performed on all animals once prior to administration of the test substance (prior to study day 0) and from all animals during study weeks 5, 9 and 13. Ophthalmic examinations were performed pre-exposure and pre-sacrifice. A gross necropsy was performed, organ weights were taken, and micropathology was performed.

At 3600/2400 ppm

Compound-related clinical findings were: unsteady and stiff back legs and lower back on study days 44, 53, and 54 in one male and unsteady and stiff back legs and lower back on study day 44 for one female. Beginning study week 9, the 3600 ppm dose group was lowered to 2400 ppm. There was a compound-related reduction in body weight for males and females during the first week of the study (- 11% and - 13%, respectively). During the first ten days of the study for males and during the first seven days of the study for females, there were statistically significant and compound-related reductions in food consumption. There was a compound-related increase in creatine phosphokinase, aspartate aminotransferase, and alanine aminotransferase at the 2-month test interval in both sexes.

There was a compound-related reduction in red blood cell count, hemoglobin, and hematocrit during the 1, 2, and 3 month hematology evaluations in both sexes. There was a compound-related increase in liver weights (absolute and relative) for males and females (14% and 11% increase in absolute weights and 28% and 24% increase in relative weights for males and females, respectively). There was also a compound-related increase in kidney weights (relative) for males (31%) and for females (16%). Microscopic pathology revealed minimal brown pigments in Kupffer cells in the liver in females and minimal to slight myofiber atrophy/degeneration in skeletal muscle in both sexes.

At 1200 ppm:

There was a compound-related decrease in body weight for males (-9%). During the first ten days of the study for males, there were statistically significant and compound-related reductions in food consumption. There was a compound-related increase in creatine Phosphokinase (CK), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) at the 2-month test interval in both sexes. There was a compound-related increase in kidney weights (relative) for males (18%). Microscopic pathology revealed myofiber atrophy/degeneration in skeletal muscle in males (minimal) and females (minimal and slight).

At 400 ppm:
No compound-related effects were observed.

In conclusion, the No Observed Adverse Effect Level (NOAEL) was 400 ppm for males and females (12 mg/kg/day).

I. Materials and Methods

A. Material

1. Test Material:	BYI02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable in diet at 100 and 5000 ppm over 7 days of storage at ambient temperature and over 35 days of storage at freezing conditions
2. Vehicle and /or positive control:	Corn oil and acetone were used to add the test compound to the diet
3. Test animals:	
Species:	Dog (Canis familiaris)
Strain:	Beagle (nulliparous and non pregnant)
Age:	7 to 8 months approximately
Weight at dosing:	7.9 to 9.8 kg for males 6.0 to 7.7 kg for females.
Source:	[redacted]
Acclimation period:	11 days
Diet:	Purina Mills Lab Canine Diet Etts 5006-3 was available for *ad libitum* consumption except when animals were fasted prior to bleeding
Water:	Tap water provided continuously for *ad libitum* consumption. The water was sampled monthly by the Kansas City Missouri Water Department and analyzed for a variety of potential impurities (*e.g.*, aflatoxins, chlorinated hydrocarbons, heavy metals, etc.).
Housing:	Individually housed in stainless steel runs

Environmental condition:
Temperature: 18 °C to 29 °C
Humidity: 30 to 70 %
Air changes: Averaged at at least 17.06 changes per hour during the dosing period
Photoperiod: Alternating 12-hour light and dark cycles. Three times during the study the lights went off during the photo period due to power outages (approximately 1.5 hours on one occasion and approximately 3 hours on two occasions).

B. Study Design and methods

1. In life dates

14 April to 15 July 2009 at [redacted], USA.

2. Animal assignment and treatment

The dogs were randomly assigned to dose groups, based on weight, using INSTEM DATATOX®. Weight variation of animals used was targeted not to exceed +20% of the mean weight for each sex. All dogs arrived at the test facility with a supplier's identification number tattooed on the inner part of the ear. This unique identifier was cross-referenced with the unique identification number assigned to each animal.

The animal care procedures and room conditions during this study were in accordance with accepted standards of laboratory animal care in compliance with the *Animal Welfare Act of 1966* (Public Law 89-544) and its amendments, as well as the *Guide for the Care and Use of Laboratory Animals,* (institute of Laboratory Animal Resources, National Academy Press, 1996), and should not have affected the outcome of the study. The facility in which the study was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) in addition to being a registered research facility with the United States Department of Agriculture (USDA).

All dogs in the study were administered control diet (diet mixed with corn oil and acetone) or Technical Grade BYI 02960 at nominal concentrations of 400, 1200, or 3600/2400 ppm in the diet for at least 90 days.

Table 5.3.3-01: Study design

Test group	Concentration in diet (ppm)	Dose per animal (average) *		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	4	4
2	400	12	12	4	4
3	1 200	33	41	4	4
4	3 600/2 400	102/85	107/78	4	4

* The mg/kg/day dose was calculated using the analytical concentration of BYI 02960 in the diet and the following equation: average daily food consumption per week/average body weight per week x ppm in the feed/1000.
The average body weight/week = (mean weight at the beginning of the week + mean weight at the end of the week)/2.

3. Diet preparation and analysis
All feed mixtures were prepared weekly by mixing appropriate amounts of the test substance with Purina Certified Canine Diet 5006-3 and then stored under freezer conditions until presented to the animals. Corn oil, at 1% by weight of the diet, along with acetone was used as vehicles to dissolved/suspend the test substance prior to being mixed in the diet. The control diet was prepared the same as the treated diet, excluding only the test substance. A sample of each batch of feed mixed was taken and retained under freezer conditions until the study was complete and the study data deemed satisfactory.
The homogeneity, room temperature stability and freezer stability were checked at 100 and 5000 ppm:. The mean technical concentrations for the homogeneity checks were 96% to 100% of the nominal concentration, and therefore within target ranges. After 7 days at room temperature there was no decline in technical concentration for the 100 ppm or 5000 ppm level. Both study levels were below the 15% loss acceptance criteria for stability. After 35 days in the freezer there was a 1% decline in the 100 ppm level and no measurable decline in technical concentration for the 5000 ppm level.
The active ingredient content of the BYI 02960 in canine ration was determined. The concentration of the active ingredient in the diet was verified for weeks 1, 2, 3, 6, 11, and 14. The mean concentrations for weeks 1 through 14 for the study were 98 to 103% of the overall nominal levels. %RSD values ranged from 2.3 to 5.6. These all were within the 85 to 115% acceptance criterion for a treated ration sample.

4. Statistics
Statistical significance was determined at $p < 0.05$ for all tests with the exception of Bartlett's test, in which a probability value of $p < 0.001$ was used. All tests were two-tailed, except for gross and histopathological lesion evaluations that were one-tailed. Continuous data were analyzed by Bartlett's test for homogeneity. If the data were homogeneous an ANOVA was performed, followed by Student's t-test on parameters showing a significant effect by ANOVA. If the data were non-homogeneous a Kruskal-Wallis ANOVA was performed, followed by the Mann-Whitney U-test to identify statistical significance between groups. Frequency data, that were examined statistically, were initially analyzed by a Chi-Square procedure. If there was statistical significance using the Chi-square test, each treatment group was compared to the control group using a Fisher's Exact test.
Statistical analyses were conducted using INSTEM DATATOX® [1]

C. Methods

1. Observations
All study animals were observed at least twice daily (a.m. and p.m.) for clinical signs of toxicity (except weekends and holidays when animals were observed only once a day). Detailed clinical observations (physical examination) were performed on all animals beginning on study day -7 and were conducted on a weekly basis thereafter.

2. Body weight
Individual body weights were measured weekly throughout the study. Body weights were also taken immediately prior to necropsy to allow for calculation of organ-to-body weight ratios.

3. Food consumption and compound intake
Each animal's food consumption was measured daily throughout the study.

4. Ophthalmic examination
Following the acclimation period and prior to initiation of dosing, ophthalmic examinations were conducted on all animals. Ophthalmic examinations were also conducted on all animals sacrificed just prior to termination of the study (on study day 86).

5. Clinical chemistry
Clinical chemistry and hematology evaluations were performed on all animals (pre-exposure and during study weeks 5, 9, and 13). Animals were fasted overnight prior to the collection of blood, which was drawn via jugular venipuncture.
Red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation, platelet count, blood cell morphology, red blood cell distribution width and hemoglobin distribution width were assayed. Prothrombin time and activated partial thromboplastin time were also assayed.
Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus concentrations and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, creatine phosphokinase and lactic acid dehydrogenase activities were assayed on plasma samples, total protein, albumin, globulins and uric acid concentrations were assayed on serum samples. Albumin/globulin ratio values were calculated.

6. Urinalysis
Urinalysis was performed on all animals once prior to administration of the test substance and on all animals during study weeks five, nine and thirteen. Urine volume was collected over a 24 hour period. Any significant change in the general appearance of the urine was recorded and pH and specific gravity were assayed.
The following parameters were assayed: glucose, bilirubin, ketones, blood, protein, nitrites, leukocytes and urobilinogen. Microscopic examination of the urinary sediment was performed.

7. Sacrifice and pathology
On study days 91, 92 or 93, a complete necropsy was performed on all animals. Animals were deeply anaesthetized by intravenous injection of Fatal-Plus and then exsanguinated before necropsy. The necropsy consisted of a systematic gross examination of each animal's general physical condition, body orifices, external and internal organs and tissues. Adrenal gland, brain, epididymides, heart, kidney, liver (with gall bladder), lung, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed fresh.
The following organs or tissues were sampled: adrenal gland, aorta, bone (sternum, rib), bone marrow, brain, epididymis, oesophagus, eye and optic nerve, Fallopian tube, heart, intestine (duodenum,

jejunum, ileum, caecum, colon, rectum), kidney, larynx/nasopharynx, liver with gall bladder, lung, lymph nodes, mammary gland, ovary, pancreas, pituitary gland, prostate gland, salivary gland, sciatic nerve, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, testis, thymus, thyroid gland (with parathyroid), trachea, urinary bladder, uterus (with cervix), ureter and vagina.
All of the above tissues were preserved in 10% buffered formalin with the exception of the eyes and optic nerves that were preserved in Davidson's fixative and testes and ovaries that were preserved in Bouin's fixative.
All tissues from the control and high-dose (3600/2400 ppm) group were processed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under the light microscope. As there were findings in the liver and muscle in the high-dose group, the mid- and low-dose groups were evaluated. Where appropriate, all findings were assigned a severity score of normal, minimal, mild or slight, moderate, and marked.

II. Results and discussion

A. Observations

1. Mortality

No animals were found dead or sacrificed *in-extremis* in this study.

2. Clinical signs of toxicity

The only compound-related clinical findings occurred in high-dose group (3600/2400 ppm) dogs. Compound-related clinical findings were: unsteady and stiff back legs and lower back observed in one male on study days 44, 53, and 54; and unsteady and stiff back legs and lower back observed in one female on study day 44. Beginning study week 9, the high-dose was lowered from 3600 ppm to 2400 ppm.

B. Body weight and body weight gain

There was a compound-related reduction in weight during the study for the 1200 and 3600/2400 ppm dose group males and the 3600/2400 ppm dose group females, beginning during the first week of the study.

Table 5.3.3-02: Significant effects in body weight and body weight gain

BYI 02960 Dose level (ppm)	0	400	1200	3600/2400
Male				
Initial BW (Day 0) (%C)	**9072**	**9046 (99%)**	**8880 (98%)**	**9233 (102%)**
BW Week 1 (Day 7) (%C)	9260	9410 (102%)	8931 (96%)	8981 (97%)
BW Week 8 (Day 56) (%C)	9543	9978* (105%)	8766 (92%)	8231* (86%)
BW Week 9 (Day 63) (%C)	9881	10370 (105%)	9146 (93%)	8695* (88%)
Final BW Week 12 (Day 84) (%C)	**9848**	**10328 (105%)**	**8940 (91%)**	**8812 (89%)**
BWG Weeks 0-1 (Days 0 to 7) (%C)	188.7	364.0	50.3	-252.0
BWG Weeks 0-9 (Days 0 to 63) (%C)	809.7	1324.5	265.5	- 538
BWG Weeks 9-12 (Days 63 to 84) (%C)	- 33.2	- 42.7	- 205.5	-117
Overall BWG (Days 0 to 84) (%C)	**777**	**1282 (140%)**	**60* (8%)**	**- 421***
Female				
Initial BW (Day 0) (%C)	**6770**	**6958 (103%)**	**6842 (101%)**	**7021 (104%)**
BW Week 1 (Day 7) (%C)	7271	7099 (98%)	6939 (95%)	6816 (94%)
BW Week 8 (Day 56) (%C)	7733	7555 (98%)	7395 (96%)	6531 (84%)
BW Week 9 (Day 63) (%C)	8011	8016 (100%)	7652 (96%)	6952 (87%)
Final BW Week 12 (Day 84) (%C)	**8095**	**7721 (95%)**	**7513 (93%)**	**7022 (87%)**
BWG Weeks 0-1 (Days 0 to 7)	500.7	140.5	96.8	- 205.3
BWG Weeks 0-9 (Days 0 to 63)	1241	1058	810.3	- 69.3
BWG Weeks 9-12 (Days 63 to 84)	84	294.5	- 139.5	70.0
Overall BWG (Days 0 to 84) (%C)	**1325.0**	**774.8 (59%)**	**670.8 (51%)**	**0.8* (0%)**

* : Statistical significantly different from the control group at $p \leq 0.05$

C. Food consumption and compound intake

During the first ten days of the study for males in the 1200 and 3600/2400 ppm dose groups, and during the first seven day of the study for females in the 3600/2400 ppm dose group, there were statistically significant and compound-related reductions in food consumption. Although not statistically significant, lower food consumption was observed in the females in the 3600/2400 ppm dose group during the first few weeks of the study.

Table 5.3.3-03: Significant effects in food consumption

BYI 02960 Dose level (ppm)	0	400	1200	3600/2400
Male				
FC Week 1 (Day 1 to 7) (%C)	326.5	338.5 (104%)	211.9(65%)	228.2(70%)
FC Week 2 (Day 8 to 14) (%C)	292.9	348.4 (119%)	228.7(78%)	217.4(74%)
FC Week 3 (Day 15 to 21) (%C)	268.7	296.1 (110%)	234.8(87%)	254.5(95%)
FC Week 4 (Day 22 to 28) (%C)	271.4	282.8 (104%)	219.9(81%)	237.4(87%)
Female				
FC Week 1 (Day 1 to 7) (%C)	350.1	232.5 (66%)	241.0 (69%)	169.6 (48%)
FC Week 2 (Day 8 to 14) (%C)	306.8	240.1 (78%)	251.1 (82%)	220.1 (72%)
FC Week 3 (Day 15 to 21) (%C)	304.9	223.8 (73%)	229.8 (75%)	216.2 (71%)
FC Week 4 (Day 22 to 28) (%C)	266.1	250.3 (94%)	241.7 (91%)	197.1 (74%)
FC Week 5 (Day 29 to 35) (%C)	290.9	234.3 (81%)	267.8 (92%)	232 (80%)
FC Week 6 (Day 36 to 42) (%C)	262.9	233.4 (89%)	248.7 (95%)	209.6 (80%)

The mean achieved dosage intake per group was: 12, 33 and 102/85 mg/kg/day in males and 12, 41 and 107/78 mg/kg/day in females.

D. Ophthalmic examination

No treatment-related ocular abnormalities were observed at ophthalmic examination.

E. Blood analysis

1. Haematological findings

In the 3600/2400 ppm dose group for males and females, there was a compound-related reduction in red blood cells (RBC), hemoglobin (Hgb), and hematocrit (Hct) during the 1, 2, and 3 month hematology evaluations. The statistically significantly lower RBC value for females in the 1200 ppm dose group on study day 56 is not considered to be compound-related as a concurrent significant reduction for Hgb and Hct was not observed. There were various other statistically significant values, but these values were not considered to be dose-related.

Table 5.3.3-04: Significant hematological changes

Dose level ppm	RBC (10^6/mm^3)				Hgb (g/dL)				Hct (%)			
	D 7	D 28	D 56	D 84	D 7	D 28	D 56	D 84	D 7	D 28	D 56	D 84
Male												
0	6.99	7.02	6.92	7.07	16.3	16.4	16.1	16.3	47.5	47.2	46.4	47.6
400	7.28	7.13	7.18	7.36	17.1	16.5	16.8	17.0	50.0	47.7	48.0	49.5
1200	6.88	6.85	6.67	6.97	15.7	15.6	15.1	15.5	45.5	44.3	43.2	45.4
3600/2400	7.5	6.64	6.29	6.75	16.8	14.9*	14.0*	14.9	49.6	42.3*	39.9*	43.2*
Female												
0	6.81	6.91	6.95	6.80	15.7	16.0	16.4	15.9	45.5	45.8	46.2	45.3
400	7.10	6.99	7.25	7.31	15.9	15.8	16.6	16.9	46.0	45.5	47.6	48.3
1200	6.65	6.41	6.44*	6.53	15.7	14.9	15.3	15.2	44.6	42.7	43.0	43.6
3600/2400	7.03	6.42	5.84*	6.25	16.5	14.7	13.0*	14.1	47.6	42.3	37.7*	40.7

* : Statistically different ($p \leq 0.05$) from the control

2. Clinical chemistry findings

For the males and females in the 1200 and 3600/2400 ppm dose groups, there was a compound related increase in CK, AST, and ALT at the 2-month test interval. There was no correlation between elevated plasma enzyme levels and clinical signs. The values for CK, AST and ALT were normal at the 3 month evaluation. There were other sporadic statistically significant changes that were not considered to be compound-related because the changes were either not in a dose-related manner, the values were within the range of historical control values for the parameter, or were similar to pretreatment values.

Table 5.3.3-05: Significant clinical chemistry changes on study day 56

Dose level (ppm)	0	400	1200	3600/2400
Male				
Creatine phosphokinase (U/L)	242	248	2834 §	2944 §
Aspartate aminotransferase (U/L)	38	36	241	266
Alanine aminotransferase (U/L)	30	36	221	311
Female				
Creatine phosphokinase (U/L)	282	175 *	1538 §	4836 *
Aspartate aminotransferase (U/L)	42	31 *	130 *	370 *
Alanine aminotransferase (U/L)	28	27	151 *	552 *

* : Statistically different ($p \leq 0.05$) from the control.
§ : Not statistically significant due to a large SD from dogs that had normal values.

F. Urinalysis

There were no compound-related urinalysis findings.

G. Sacrifice and pathology

1. Gross pathology

There were no gross pathology findings.

2. Organ weight

There was a compound-related increase in liver weights (absolute and relative) for male and females in the 3600/2400 ppm dose groups (14% and 11% increase in absolute weights and 28% and 24% increase in relative weights for males and females, respectively). There was also a compound-related increase in kidney weights (relative) for males in the 1200 ppm and 3600/2400 ppm dose groups (18% and 31%, respectively) and for females in the 3600/2400 ppm dose group (16%).

The statistical increase in the prostate weight for the 3600/2400 ppm dose group males is not considered compound-related as these were young dogs and variations in prostate weight due to maturation can be expected. The statistical significant values for relative lung weight in the 400 and 1200 ppm male groups are not considered to be compound-related as the lung weight was statistically significantly lower for the 400 ppm dose group and was statistically significantly higher for the 1200 ppm dose group.

Table 5.3.3-06: Significant organ weight changes

Sex	Male				Female			
BYI 02960 Dose level (ppm)	**0**	**400**	**1200**	**3600/ 2400**	**0**	**400**	**1200**	**3600/ 2400**
Liver								
Absolute liver weight (g)	269 ± 21.7	260 ± 12.8 (- 3%)	261 ± 35.7 (- 3%)	307 * ± 15.4 (+ 14%)	226 ± 27.0	201 ± 41.8 (-11%)	225 ± 22.4 (0%)	252 ± 41.3 (+ 12%)
Liver to body weight ratio (%)	2.8 ± 0.26	2.5 ± 0.10 (- 10%)	2.9 ± 0.34 (+ 4%)	3.5* ± 0.55 (+ 20%)	2.9 ± 0.26	2.6 ± 0.28 (-10%)	3.0 ± 0.09 (+ 3%)	3.6* ± 0..35 (+ 24%)
Kidney								
Absolute kidney weight (g)	47 ± 1.1	55 ± 5.3 (+17%)	51 ± 8.1 (+9%)	55 ± 2.3 (+17%)	38 ± 2.8	34 ± 5.6 (-11%)	36 ±2.3 (-5%)	38 ± 2.5 (0%)
Kidney to body weight ratio (%)	0.49 ± 0.02	0.54 ± 0.06 (+ 10%)	0.57* ± 0.05 (+16%)	0.63* ± 0.10 (+ 29%)	0.47 ± 0.01	0.44 ± 0.07 (-6 %)	0.49 ± 0.06 (+ 4%)	0.55 ± 0.03 (+ 17%)

3. Microscopy

In the liver, Kupffer cells brown pigments were observed at the microscopic level in two females at 3600/2400 ppm.

In the skeletal muscle, myofiber atrophy/degeneration was noted in both sexes at 3600/2400 and 1200 ppm.

Table 5.3.3-07: Significant microscopic findings

Sex	Male				Female			
BYI 02960 Dose level (ppm)	0	400	1200	3600/ 2400	0	400	1200	3600/ 2400
Number of animals examined	4	4	4	4	4	4	4	4
Myofiber atrophy/degeneration: focal								
Minimal	0	0	2	1	0	0	3	4
Slight	0	0	0	1	0	0	1	0
Total	**0**	**0**	**2**	**2**	**0**	**0**	**4**	**4**

III. Conclusions

In conclusion, the NOAEL was 400 ppm for males and females (12 mg/kg/day).

KIIA 5.3.4 - Oral 1 year toxicity (dog)

Report:	**KIIA 5.3.4/01; [redacted]; 2011;**
Title:	A Chronic Toxicity Feeding Study in the Beagle Dog with Technical Grade BYI 02960
Report No & Document No	09-C76-RZ M-425272-03-1
Guidelines: Deviations:	OECD 452 (2009); EPA Health Effects Test Guideline (OPPTS 870.4100; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines. None
GLP	Yes (certified laboratory)

Executive Summary

BYI 02960 (batch number 2009-000239, 96.2% purity) was administered to Beagle dogs of both sexes by dietary administration to evaluate its potential chronic toxicity.

Male and female Beagle dogs (4/sex/dietary level) were fed control feed or feed containing BYI 02960 at dietary concentrations of 150, 300, or 1000 ppm (approximately equal to 4.6/4.1, 7.8/7.8, 28.1/28.2 mg/kg body weight/day in males/females, respectively) for at least one year. All animals were observed at least twice daily (a.m. and p.m.) for clinical signs of toxicity (except weekends and holidays when animals were observed only once daily). Detailed clinical observations (physical examination) were performed on all animals on a weekly basis. Food consumption was measured daily, and body weights were measured weekly. Ophthalmic exams were conducted on all animals prior to initiation of dosing and just prior to necropsy. Clinical chemistry, hematology, and urinalysis evaluations were performed on all animals once prior to administration of the test substance and at approximately 3, 6, 9, and 12 months during the treatment period. A gross necropsy was conducted on all animals, selected organ weights were taken, and a range of tissues were collected and processed for histopathological examination.

Dietary administration of BYI 02960 to male and female Beagle dogs at 150, 300, or 1000 ppm for at least 52 weeks did not result in mortalities, clinical signs, changes in body weight or food consumption, changes at the physical and ophthalmologic examinations, changes in clinical pathology (clinical chemistry, hematology and coagulation, urinalysis), gross lesions, or changes in organ weights attributable to the test substance.

No effects attributable to the test substance were observed at 150 and 300 ppm.

Test substance-related effects were limited to degeneration noted in skeletal muscle (gastrocnemius, biceps femoris) of males and females at 1000 ppm only. Minimal to slight, focal to multifocal areas of degeneration of skeletal muscles were noted in males (gastrocnemius - incidence 2/4; biceps femoris - incidence 3/4) and females (gastrocnemius - incidence 2/4; biceps femoris - incidence 3/4). Degeneration of the myofiber comprised one or more of the following changes: atrophy, necrosis, and/or presence of inflammatory cells around the affected myofiber. Skeletal muscles evaluated from 150 ppm and 300 ppm males and females were comparable to controls. All other microscopic findings were considered to be incidental and unrelated to treatment.

No other effects attributable to exposure to the test substance were observed.

Based on the micropathology findings, the lowest-observed-adverse-effect-level (LOAEL) in this study was 1000 ppm, which was equivalent to 28.1 and 28.2 mg/kg body weight/day for male and female dogs, respectively. Based on the lack of adverse compound-related effects, a dietary level of 300 ppm (equivalent to 7.8 mg/kg body weight/day for both sexes) was considered to be a no-observed-adverse-effect-level (NOAEL).

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable in diet at 100 and 5000 ppm over 7 days of storage at ambient temperature and over 35 days of storage at freezing conditions
2. Vehicle and/or positive control:	Corn oil and acetone were used to add the test material to the diet
3. Test animals:	
Species:	Dog (Canis familiaris)
Strain:	Purebred Beagle (nulliparous and non-pregnant)
Age:	5-6 months
Weight at dosing:	7.9 to 9.8 kg for males 6.0 to 7.7 kg for females.
Source:	[redacted]
Acclimation period:	11 days
Diet:	Purina Mills Lab Canine Diet Etts 5006-3 was available for ad libitum consumption except when animals were fasted prior to bleeding
Water:	Tap water was provided continuously for *ad libitum* consumption. The water was sampled monthly by the Kansas City Missouri Water Department and analyzed for a variety of potential impurities (*e.g.*, aflatoxins, chlorinated hydrocarbons, heavy metals, etc.).
Housing:	Individually housed in stainless steel runs

Environmental condition:	Temperature:	18 °C to 29 °C
	Humidity:	30 to 70 %
	Air changes:	Averaged at least 17.06 changes per hour during the dosing period
	Photoperiod:	Alternating 12-hour light and dark cycles. Three times during the study the lights went off during the photo period due to power outages (approximately 1.5 hours on one occasion and approximately 3 hours on two occasions).
	Acclimatization:	At least 6 days

B. Study Design and methods

1. In life dates

Dosing from 26 January 2010 to 26 - 28 January 2011

2. Animal assignment and treatment

Upon receipt, all dogs were examined by a veterinarian and were subjected to a detailed clinical examination to assess their general health, behavior, and gross external abnormalities. Only animals free of ill health were placed on study. During the acclimatization phase, animals were checked twice daily for moribundity and mortality except on weekends and public holidays, during which time they were checked once daily.

Animals were allocated to dosage groups using a dedicated computer system (Path/Tox System version 4.2.2) in order to ensure a similar body weight distribution among groups of each sex while ensuring full siblings were not placed in the same treatment group. Animals were assigned to the test groups noted in the following table. Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 5.3.4-01: Test groups, dose levels and achieved dosages**

Test Group (Group number)	Nominal Concentration of BYI 02960 in Diet (ppm)	Mean Dietary Concentration of BYI 02960 (ppm)	Achieved dose to animal (Weeks 1 - 52) (mg/kg body weight/day)
Control (1)	0	Not Detected	0
Low (2)	150	145 ± 5	M: 4.6 F: 4.1
Mid (3)	300	290 ± 14	M: 7.8 F: 7.8
High (4)	1000	975 ± 33	M: 28.1 F: 28.2

** : All animals survived the duration of the main study (12 months)

Diet Preparation and Analysis

Test substance formulations were prepared at least weekly and were used to cover the dietary requirements for one week. Technical grade BYI 02960 was administered at a constant concentration in the feed for the duration of the study. Adjustments were not made for percentage purity of less than 100%. Corn oil and acetone were used to dissolve BYI 02960 for addition to the feed.

BYI 02960 was dissolved with acetone and corn oil in an Erlenmeyer flask. The test substance mixture was then transferred to a separatory funnel and added to the diet while the diet was being mixed with a Hobart mixer. After the test substance mixture was added to the diet, the Erlenmeyer flask and separatory funnel were rinsed with acetone and the rinse added to the ration. The acetone carrier evaporated during the 10-minute mixing time. The control diet was prepared in the same manner as the chemically treated diet excluding only the test substance.

The homogeneity and the stability of the test substance in feed (containing corn oil and acetone) stored at room temperature for 7 days and freezer temperature for 35 days were determined (M-369978-01-2). The concentration of the active ingredient in the feed was verified weekly for the first three weeks of the study and monthly thereafter. Samples of each batch of feed mixed were retained in a freezer for subsequent analysis, if deemed appropriate. These analyses were conducted by Bayer CropScience LP, Analytical and Bioanalytical, Residue, and Environmental Chemistry Group, Stilwell KS using a validated method (method number BCSM-BYI60-732666673-002).

Feed samples (at least 100 g) were collected by [redacted] personnel at the test facility and transferred (hand-carried, under ambient conditions) to the test site for concentration analysis.

3. Statistics

Statistical significance was determined at $p < 0.05$ for all tests with the exception of Bartlett's test, in which a probability value of $p < 0.001$ was used. All tests were two-tailed, except for histopathologic lesion evaluations that were one-tailed.

Statistical analyses were carried out using DATATOX software, Version rC.10, and SAS software, (SAS Institute Inc., Version 6.09 Enhanced, Cary, North Carolina). SAS software was used only for micropathology incidence evaluation and DATATOX was used for all other data. Continuous data were analyzed by Bartlett's test for homogeneity of variances. These data were found to be homogeneous and subsequently had an Analysis of Variance (ANOVA) performed followed by Student's *t*-Test on parameters showing a significant effect by ANOVA.

Frequency data (*i.e.* micropathology incidence, etc.), that are examined statistically were initially analyzed by a chi-square test. If statistical significance was achieved using the chi-square test, each treatment group was compared to the control group using a Fisher's exact test.

C. Methods

1. Observations

Clinical signs and mortality

Observed clinical signs were recorded at least twice daily (a.m. and p.m.) during the week and at least once daily on weekends and holidays. Any deviation from normal was recorded in respect to the nature and severity. Daily examination of the kennels also recorded observations for vomitus, diarrhea or blood.

Detailed clinical examinations (physical examination) were performed in an open area on all animals beginning on the day treatment was initiated and approximately weekly thereafter throughout the study.

Recording of clinical signs included but was not limited to changes in: general behavior and appearance, skin and fur, teeth and gum, eyes, ears, mucous membranes, gait, posture and response to handling.

Each animal was checked for evidence of behavioral changes, ill-health, moribundity and mortality twice daily or once daily on weekends and public holidays throughout the study. Any animal suffering from severe distress, in moribund condition or considered unlikely to survive was sacrificed and necropsied.

Physical examination

A detailed physical examination was performed on all dogs weekly following arrival until sacrifice. The physical examination included but was not necessarily restricted to the following examinations: fur and skin, eyes, ears, teeth, gum, mucous membranes, gait, stance, general behavior, chest including respiratory rate, abdomen including palpitation, external genitalia and mammary glands.
The following parameters were also evaluated during the physical examination:

- Posture
- Gait and motor function
- Muscle tone
- Mental state
- Level of consciousness
- Behavioral change
- General examination of the head

2. Body weight

Individual body weights were recorded weekly throughout the treatment. Body weights were also taken immediately prior to necropsy to allow for calculation of organ-to-body weight ratios.

3. Food consumption

Food intake was measured for a minimum of five consecutive days immediately before start of treatment. The weight of food supplied to each animal and that remaining were recorded daily throughout the treatment period. From these records, the mean weekly consumption was calculated for each dog. Food spillage was also recorded.

The group mean achieved dosage for each sex, expressed as mg/kg body weight/day, was calculated for each week and the overall mean subsequently derived for weeks 1 to 52.

4. Ophthalmic examination

Following the acclimation period and prior to initiation of dosing, ophthalmic examinations were conducted on all animals. Ophthalmic examinations were also conducted on all animals just prior to termination of the study.

5. Hematology and clinical chemistry

Clinical chemistry and hematology were performed on all animals once prior to administration of the test substance and at approximately 3, 6, 9, and 12 months during the treatment period. Animals were

fasted overnight prior to the collection of blood (approximately 8 mL), which was drawn via jugular venipuncture. The blood was collected directly into Vacuette® or similar tubes containing potassium EDTA for hematology, serum separator tubes for clinical chemistry, and sodium citrate for coagulation.

Hematological parameters were measured using an Advia 120 (Siemens, Eragny, France) and an ACL Elite Pro (Instrumentation Laboratory, Paris, France) for blood clotting measurements. Any significant change in the general appearance of the plasma and the serum was recorded. Clinical chemistry parameters were measured using an Advia 1650 (Siemens, Eragny, France).

The following hematology parameters were evaluated in this study:

X	Blood Cell Morphology	X	Leukocyte differential count*
X	Hematocrit*	X	Mean corpuscular hemoglobin*
X	Hemoglobin concentration*	X	Mean corpuscular volume*
X	White blood cell count*	X	Reticulocyte count, % Reticulocytes
X	Red blood cell count*	X	Mean corpuscular hemoglobin concentration*
X	Platelet count*	X	Activated Partial Thromboplastin Time
	Blood clotting measurements	X	Prothrombin time
X	Activated partial thromboplastin time	X	Hemoglobin Distribution Width
		X	Red Blood Cell Distribution Width

* : recommended for chronic toxicity based on OPPTS Guideline 870.4100
(X) : Parameters examined in serum samples

The following clinical chemistry parameters were measured:

	Electrolytes		**Others**
XX	Calcium*	X	Albumin* Albumin/Globulin ratio
XX	Chloride*	XX	Creatinine*
XX	Inorganic phosphorus*	XX	Urea*
XX	Potassium*	XX	Total Cholesterol*
XX	Sodium*	XX	Glucose*
		XX	Total bilirubin*
	Enzymes (more than 2 hepatic enzymes)	X	Total protein*
XX	Alanine aminotransferase	XX	Triglycerides
XX	Aspartate aminotransferase	XX	Creatinine phosphokinase
XX	Alkaline phosphatase	X	Globulin
XX	Gamma glutamyltransferase	XX	Uric Acid

* : recommended for chronic toxicity based on OPPTS Guideline 870.4100
(X) : Parameters examined in serum samples
(XX) : Parameters examined in plasma samples

6. Urinalysis

Urinalysis (including parameters measured in the table below) was performed on all animals once prior to administration of the test substance and on all animals at approximately 3, 6, 9, and 12 months during the treatment period. Urine was collected by placing a metabolism pan under each animal's run over a 24 hour period.

X	Appearance*	X	Glucose*
X	Volume (24-hour)*	X	Ketones
X	Specific gravity / osmolality / refractive index*	X	Bilirubin
X	pH*	X	Blood / red blood cells*
X	Sediment (microscopic)	X	Urobilinogen
X	Protein*		

* : recommended for chronic toxicity based on OPPTS Guideline 870.4100

7. Sacrifice and Pathology

On study days 365-368, all animals were sacrificed by intravenous injection of Fatal-Plus®. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically.

The following organs or tissues were sampled and/or weighed at necropsy:

	Digestive system		Cardiav ASC./Hemat.		Neurologic
		X	Aorta*	XX XX	Brain* Cerebellum*
X	Submaxillary (salivary) gland*	XX	Heart*	X	Sciatic nerve*
X	Esophagus*	X	Bone marrow, sternum*	X	Spinal cord (cervical, thoracic, lumbar)*
X	Stomach*	X	Lymph node, mesenteric*	X	Eyes*
X	Duodenum*	X	Lymph node, retropharyngeal	X	Optic nerves*
X	Jejunum*	XX	Spleen*	XX	Pituitary gland*
X	Ileum*	XX	Thymus*		
X	Cecum*				GLANDULAR
X	Colon*		UROGENITAL	XX	Adrenal gland*
X	Rectum*	XX	Kidney*	X	Lacrymal exorbital gland*
XX	Liver (with gall bladder)*	X	Urinary bladder*	X	Parathyroid gland*
X	Pancreas*	XX	Testis*	XX	Thyroid gland* (weighed with parathyroid gland)
		XX	Epididymis*	X	Harderian gland
	Respiratory	XX	Prostate gland*		
X	Trachea*	X	Seminal vesicle*		other
X	Lung*	XX	Ovary*	X	Bone (sternum, rib)
X	Nasal cavities*	XX XX	Uterus* Cervix*	X	Skeletal muscle
X	Pharynx*	X	Mammary gland*	X	Skin*
X	Larynx*	X	Vagina	X	All gross lesions and masses
		X	Oviduct	X	Articular surface (femorotibial joint)

*: recommended for chronic toxicity based on OPPTS Guideline 870.4100
(X): Tissues were collected
(XX): Organs were weighed fresh at scheduled sacrifice. Paired organs were weighed together

All of the above tissues were preserved in 10% buffered formalin with the exception of the eyes and optic nerves which were preserved in Davidson's fixative, and testes and ovaries, which were preserved in Bouin's fixative. Histopathological examinations were performed on all tissues from all the animals in all dose groups.

II. Results and discussion

A. Observations

1. Clinical signs of toxicity

At 150, 300, and 1000 ppm, no treatment-related clinical signs or changes at physical examination were noted in either sex.

2. Mortality

Survival was unaffected by the test substance. All animals survived to their scheduled sacrifice.

B. Body weight and body weight gain

Body weights measured weekly were not statistically significantly affected by the test substance. Body weight gains over the course of the study were affected in females at 1000 ppm. Despite an average group starting weight approximately 200 g higher than controls, females at this dose level gained 9% less weight relative to the control weight at the end of the study (Single factor ANOVA, $p = 0.05$).

Table 5.3.4-02: Mean body weight gain and body weights (kg) in male and female dogs during the 52-week study with BYI 02960

Dosage level of BYI 02980	0 ppm	150 ppm	300 ppm	1000 ppm
Male				
Initial BW (Day 0) (%C)	**7.2 ± 0.5**	**7.0 ± 0.5 (96%)**	**7.1 ± 0.5 (99%)**	**7.0 ± 0.4 (98%)**
BWG Week 1 (Days 0-7)	0.2 ± 0.1	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.1
BWG Weeks 1-13 (Days 0-91)	2.3 ± 1.0	2.2 ± 1.3	2.7 ± 0.7	2.8 ± 0.9
BWG Weeks 13-26 (Days 91-182)	1.1 ± 0.3	1.3 ± 0.2	0.9 ± 0.3	1.6 ± 0.4
BWG Weeks 26-52 (Days 182-364)	0.3 ± 0.6	0.2 ± 0.3	0.5 ± 0.5	0.8 ± 0.5
BWG Weeks 1-52 (Days 0-364)	**3.9 ± 1.8**	**3.6 ± 1.6**	**4.2 ± 1.2**	**5.3 ± 1.6**
Final BW (Day 364) (%C)	**11.1 ± 2.1**	**10.5 ± 2.1 (95%)**	**11.3 ± 1.4 (102%)**	**12.3 ± 1.9 (110%)**
Female				
Initial BW (Day 0) (%C)	7.1 ± 0.7	7.3 ± 0.7 (103%)	7.3 ± 0.5 (103%)	7.3 ± 0.4 (103%)
BWG Week 1 (Days 0-7)	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.4	0.05 ± 0.2
BWG Weeks 1-13 (Days 0-91)	1.2 ± 0.3	1.9 ± 0.5	2.0 ± 0.5	1.4 ± 0.4
BWG Weeks 13-26 (Days 91-182)	0.9 ± 0.2	0.7 ± 0.4	0.7 ± 0.4	0.3 ± 0.4
BWG Weeks 26-52 (Days 182-364)	0.5 ± 0.4	0.4 ± 0.1	1.0 ± 0.6	0.2 ± 0.2
BWG Weeks 1-52 (Days 0-364)	**3.0 ± 0.6**	**3.0 ± 0.5**	**3.7 ± 1.0**	**1.2 ± 0.9**
Final BW (Day 364) (%C)	**10.2 ± 0.7**	**10.3 ± 1.1 (101%)**	**11.0 ± 0.7 (108%)**	**9.2 ± 0.7 (91%)**

BW: body weight BWG: body weight gain %C: % versus control

C. Food consumption

Food consumption was unaffected in both sexes at all dietary levels tested. Sporadic statistically significantly increases or decreases were not dose-related and did not represent a treatment-related effect.

Table 5.3.4-03: Mean food consumption (g/day) in male and female dogs during the 52-week study with BYI 02960

Dosage level of BYI 02980	0 ppm	150 ppm	300 ppm	1000 ppm
Male				
Week 1 (Days 0-7) (%C)	273	319 (117%)	282 (103%)	257 (94%)
Weeks 1-52 (Days 0-364) (%C)	291	319 (109%)	277 (95%)	313 (107%)
Female				
Week 1 (Days 0-7) (%C)	301	279 (93%)	285 (95%)	227 (75%)
Weeks 1-52 (Days 0-364) (%C)	255	272 (107%)	266 (104%)	260 (102%)

%C : % versus control

D. Ophthalmoscopic examination

Ophthalmic changes attributable to the test substance were not observed in this study.

E. Blood analysis

1. Haematological findings

Hematology or coagulation parameter changes attributable to the test substance were not observed in this study.

2. Clinical chemistry findings

Clinical chemistry parameter changes attributable to the test substance were not observed in this study.

3. Toxicokinetics

No BYI 02960 was detected in the blood of control animals at any time.

Blood plasma levels of BYI 02960 were low (3.00 µg/mL or less) and peaked at the 3-hour time point in animals administered the nominal dietary level of 300 ppm (Table 5.3.4-04).

Table 5.3.4-04: Plasma levels of BYI 02960 collected on study day 141 of the 52-week study with BYI 02960

Animal Number	Sex	Dose Level (ppm)	Amount of Food Eaten in One Hour Prior to Start (g)	Time (h)	BYI 02960 (µg/mL)
RZ0001	Male	0	18	1	ND
RZ0101	Female	0	70	1	ND
RZ0001	Male	0		8	ND
RZ0101	Female	0		8	ND
RZ2001	Male	300	75	1	2.57
RZ2101	Female	300	94	1	1.70
RZ2001	Male	300		3	3.00
RZ2101	Female	300		3	2.26
RZ2001	Male	300		8	1.63
RZ2101	Female	300		8	1.11

F. Urinalysis

Urinalysis parameter changes attributable to the test substance were not observed in this study.

G. Sacrifice and pathology

1. Organ weight

There were no test substance-related changes in absolute or relative organ weights observed in either sex.

2. Gross and pathology

There were no test substance-related gross lesions observed in either sex.

3. Microscopic pathology:

Test substance-related micropathology change was limited to degeneration noted in skeletal muscle, protocol (gastrocnemius); and muscle, other (biceps femoris).
The microscopic lesion of interest is included in the table below:

Table 5.3.4-05: Microscopic findings for skeletal muscle tissues after dietary administration of BYI 02960 for 52 weeks

Organ and Associated Microscopic Finding(s)	Sex and Dose Level (ppm)							
	Male				Female			
	Control	150	300	1000	Control	150	300	1000
Skeletal muscle, protocol (gastrocnemius) - Degeneration, myofiber	0	0	0	2 (1.5)	0	0	0	2 (1.0)
Muscle, other (biceps femoris) - Degeneration, myofiber	0	0	0	3 (1.3)	0	0	0	3 (1.0)

Key :Values : number of dog(s) showing finding(s)
() : Average severity of dogs with lesion; 1 (minimal) to 5 (severe)
Total number of dogs per group = 4.

Minimal to slight, focal to multifocal areas of degeneration of skeletal muscles (gastrocnemius and biceps femoris) were noted at 1000 ppm males and females only. Degeneration of the myofiber comprised of one or more of the following changes: atrophy, necrosis, and/or presence of inflammatory cells around the affected myofiber. The skeletal muscles evaluated from 150 ppm and 300 ppm males and females were comparable to controls.

All other microscopic findings were considered incidental/background.

III. Conclusions

Based on decreased body weight gains in females and micropathology findings in males and females, the LOAEL in this study was 1000 ppm, which was equivalent to 28.1 and 28.2 mg/kg body weight/day for male and female dogs, respectively. The systemic chronic toxicity NOAEL in this study was 300 ppm, equivalent to 7.8 mg/kg body weight/day for both male and female dogs.

KIIA 5.3.5 - 28-day inhalation toxicity (rodents)

Based on the results of the acute inhalation toxicity study and the physical properties of BYI 02960 (compound not volatile with an extrapolated vapour pressure of 9.1×10^{-7} Pa at 20 °C), no repeat inhalation study is triggered.

KIIA 5.3.6 - 90-day inhalation toxicity (rodents)

Based on the results of the acute inhalation toxicity study and the physical properties of BYI 02960 (compound not volatile with an extrapolated vapour pressure of 9.1×10^{-7} Pa at 20 °C), no repeat inhalation study is triggered.

KIIA 5.3.7 - Percutaneous 28-day toxicity (rodents)

Report:	**KIIA 5.3.7/01, [redacted] A.; 2012**
Title:	A Subacute Dermal Toxicity Study in Rats with Technical Grade BYI 02960
Study No & Document No	11-S22-US M-432336-01-1
Guidelines:	OECD 410 (1981); EPA Health Effects Test Guideline (OPPTS 870.3200; 1998)
GLP	Yes (certified laboratory)

Executive Summary

The principal objective of this study was to determine the dermal toxicity of technical grade BYI 02960 in a subacute toxicity study with rats. Male and female rats, 10 rats/sex/group (one control and three test substance-dosed groups) were administered vehicle or BYI 02960 (50, 150, or 500 mg/kg/day) daily by dermal application for at least 28 consecutive days (minimum of 6 h/day) and euthanized one day following the last dose. An additional 6 rats were used to test compound wetting suitability. Two rats/group were treated with 200 mg BYI 02960 using 2 mL deionized water, 0.5% methylcellulose, or corn oil. After at least 2 h of exposure, water was found to be an appropriate wetting agent and was chosen to be used to dose in this study. Examined parameters included clinical observations, mortality and moribundity checks, body weights, food consumption, eye exams, clinical pathology, gross pathology, organ weights, and microscopic pathology.
Effects attributed to exposure to BYI 02960 were as follows:

<u>At 500 mg/kg/day</u>

No adverse effects attributable to exposure to the test substance were observed.

In the first two weeks of daily dermal dosing, females had significantly lower food consumption (g/animal/day) as compared to controls. In addition, food efficiency (g/kg bw/day) was significantly lower in the first week. These findings were not considered to be biologically significant as changes were not consistently affected in a dose-related fashion, were not found in males, and did not impact clinical observations or body weights at any point.
The absolute and relative liver weights at 500 mg/kg/day males were non-statistically decreased by 6% and 9%, respectively, compared to control rats. There were no test substance-related clinical or gross or microscopic pathology changes to correlate this finding.

At 150 mg/kg/day

No adverse effects attributable to exposure to the test substance were observed.

At 50 mg/kg/day

No effects attributable to exposure to the test substance were observed.
Females dosed with 50 mg/kg/day had significantly lower food consumption in the second week as compared to controls. This finding was not considered biologically significant as changes were not consistently affected in a dose-related fashion, were not found in males, and did notimpact clinical observations or body weights at any point.

Conclusions

After 28 days of repeated dermal exposure to the test substance, the toxicological response of the rat was principally characterized by non-adverse decreases in food consumption in female rats and mild decreases in absolute and relative liver weights in males with no clinical pathology or micropathology correlates.

Based on the results of this study, the no-adverse-effect-level (NOAEL) in this study was 500 mg/kg/day for both male and female rats.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Beige powder
Lot/Batch: 2009-000239
Purity: 96.2% (Date of analysis: 14 January 2011)
CAS: 951659-40-8
Stability: Stable in the diet over a concentration range of 20-2500 ppm when stored for 7 days at room temperature and following freezer storage for a minimum of 28 days (Moore, 2010)
2. Vehicle control: Deionized water

3. Test animals:
Species/Sex: Rat/male and female (nulliparous and non-pregnant)
Strain: Wistar Han CRL: WI (HAN)
Age at study initiation: 8 weeks
Weight at randomization: 158-296 g at initiation of dosing
Source: [redacted] US
Acclimation period: 19 September 2011 (receipt) to 26 September 2011 (released for study)

Diet: *Ad libitum* (with the exception of overnight fasting prior toclinical pathology/scheduled necropsy); Purina Mills Certified Rodent Diet 5002 in "meal" form; the "Certification Profile" for Purina Mills Certified Rodent Diet 5002 provided by the vendor is kept on file at [redacted]. No contaminants were present in the food in sufficient quantities to affect the conduct or results of the study.

Water: Tap water (Kansas City, MO), *ad libitum*

Housing: Animals were housed individually in wire-bottomed stainless steel cages with a deotized cage board in the bedding trays.

Environmental condition:
- Temperature: 18 to 26 °C
- Humidity: 30 to 70%
- Air changes: Minimum daily average of 17.79 air changes per hour
- Photoperiod: Alternating 12-hour light and dark cycles (the light cycle was interrupted twice for ophthalmic exams)

B. Study Design and methods

1. In-life dates

26 September 2011 to 2 November 2011 [redacted] USA.

2. Animal assignment and treatment

Following seven days of acclimation, all rats were randomly assigned to dose groups using a weight stratification-based computer DATATOX program. On the day of distribution of animals to their dose groups (and prior to initiation of exposure), each rat on study was identified via tail tattoo. At the very least, the tattoo contained a unique number specifying the animal's sex and dose group. In addition, a laminated cage card was attached to the outside of each animal's cage. All rats not placed on study, for whatever reason, were ultimately euthanized. Table 5.3.7-01 below presents the study group design.

Table 5.3.7-01: Study group design

Group[a]	No. of Rats		BYI 02960 Dose Level (mg/kg)	Deionized Water[b] (mL)
	Male	Female		
1 Control	**10**	**10**	**0**	**2/3**
2 Low Dose	**10**	**10**	**50**	**2/3**
3 Mid Dose	**10**	**10**	**150**	**2/3**
4 High Dose	**10**	**10**	**500**	**2/3**

a Group 1 received vehicle [deionized water] only.

b On study Day 1, 3 mL of deionized water was used through the end of dosing.

3. Dose selection rationale

Dose levels were selected based on the results of previous studies conducted with this compound. In an acute dermal toxicity study, which indicated an LD_{50} greater than 2000 mg/kg body weight, no mortalities, clinical signs, changes in body weight, or gross pathological effects were observed at 2000 mg/kg body weight. In addition, a subacute oral toxicity study in rats resulted in decreases in body

weight and body weight gain at the highest dietary level of 5000 ppm. The high-dose of 500 mg/kg body weight was selected after consideration of the amount of test material that can be reliably applied to the skin.

4. Preparation and treatment of animal skin

At least one day prior to study start, the hair was removed (shaved) from the dorsal and lateral areas of the trunk of each rat using electric clippers. During the dosing period, the animals were shaved, when necessary, due to hair growth.

The first day of dosing was considered study Day 0.

The dose was applied to approximately a 3-inch by 3-inch area of shaved skin. The dose site was covered by a 3-inch by 3-inch gauze pad secured onto a dermal dosing jacket to ensure contact with the skin. The same procedure was performed for control animals, except only gauze moistened with 2 mL of deionized water was applied to the dose site.

Day 1+: The dose was applied to approximately a 3-inch by 3-inch gauze pad pre-wetted with 3 mL of deionized water. The animal was then fitted with a dermal dosing jacket and a removable strap to ensure contact with the skin. The same procedure was performed for control animals, except only gauze moistened with 3 mL of deionized water was applied to the dose site.

The test substance was held in contact with the skin for a minimum of 6 h/day for at least 28 consecutive days (with the exception of 8 instances where animals were found out of wrapping/dermal jackets at the time of unwrapping/washing; this deviation had no impact on the outcome of the study). Each day the jackets were removed and the dose site was gently wiped with water-dampened and dry gauze to remove as much test substance residue as feasible without damaging the skin.

5. Statistics

The data was collected using applications provided by DATATOX software version rC.10. The data was analyzed using applications provided by DATATOX. Statistical significance was determined at $p \leq 0.05$ for all tests with the exception of Bartlett's Test, in which a probability value of $p \leq 0.001$ was used. All tests were two-tailed except for gross and histopathologic lesion evaluations that were one-tailed.

Continuous data was analyzed by Bartlett's Test for homogeneity. If the data was homogeneous, an analysis of variance (ANOVA) was performed followed by Dunnett's *t*-Test on parameters showing a significant effect by ANOVA.

C. Methods

1. Observations

All animals were observed at least twice daily (a.m. and p.m.) for clinical signs of toxicity/mortality (except weekends and holidays when animals were observed only once a day).

Detailed clinical observations for clinical signs of toxicity were performed on all animals beginning on

the day treatment is initiated (prior to first dose administration) and approximately weekly thereafter.

2. Body weight
The non-fasted body weight of each animal was measured on study Days 0, 7, 14, 21, and 28. Body weights (fasted) were also taken immediately prior to necropsy to allow for calculation of organ to body weight ratios.

3. Food consumption
Food consumption was measured at least weekly during the study.

4. Ophthalmologic examinations
Ophthalmic exams were conducted predose and within 5 days of sacrifice The predose examination was recorded as 44 males and 44 females on a form prepared prior to animal release, when in fact 43 males and 45 females were examined. Eye exams occurred on 88 animals with all animals examined being normal. This deviation had no impact on the outcome of the study as there were no pre-dose ophthalmic findings.

5. Hematology and clinical chemistry
During the fourth week of the study, following an overnight fast, each rat was anesthetized with inhaled isoflurane and blood samples were collected from the orbital sinus using a capillary tube (~500 µL for hematology, ~1000 µL for serum chemistry, ~1800 µL for coagulation). The blood was collected into tubes appropriate for the analysis of clinical chemistry (serum, no anticoagulant), hematology (EDTA), or coagulation (sodium citrate) parameters. Uric acid was not evaluated in males; this deviation had no impact on the outcome of the study. The clinical chemistry and hematology parameters evaluated were as follows.

a. Hematology

blood cell morphology	mean corpuscular hemoglobin concentration (MCHC)
erythrocytes (RBC)	platelets (PLTS)
hematocrit (HCt)	reticulocytes (Retic)
hemoglobin (Hgb)	hemoglobin distribution width (HDW)
leukocytes (WBC) (total & differential)	red blood cell distribution width (RDW)
mean corpuscular volume (MCV)	mean corpuscular hemoglobin (MCH)
prothrombin time (PT)	activated partial thromboplastin time (APTT)

b. Clinical chemistry

alanine aminotransferase (ALT)	creatine phosphokinase (CK)
albumin (Alb)	gamma-glutamyltranspeptidase (GGT)
A/G ratio	globulin (Glob)
alkaline phosphatase (ALP)	glucose (Gluc)
aspartate aminotransferase (AST)	lactic dehydrogenase (LDH)
bilirubin, total (T-Bili)	phosphate (Phos)
blood urea nitrogen (BUN)	potassium (K)
calcium (Calc)	protein, total (T-Prot)
chloride (Cl)	sodium (Na)

cholesterol, total (Chol) triglycerides (Trig)
creatinine (Creat) uric acid (Uric-A)

6. Sacrifice and pathology

After an overnight fast, all animals were sacrificed at their scheduled termination via CO2 inhalation. A standard necropsy was conducted on all animals. Organs weighed and tissues preserved as part of the postmortem examination are listed below in Table 5.3.7-02.

Gross lesions from animals in all dose groups and representative sections of the tissues listed below for all control and high-dose group animals were processed, stained with hematoxylin and eosin (H &E), and examined under light microscope.

The following tissues (when present) was collected from each animal and preserved in 10% neutral buffered formalin, unless otherwise indicated.

Table 5.3.7-02: Tissue weight and collection

Tissue	Organ Weight Taken	Collected and Preserved	Microscopic Examination
Adrenal gland (2)	X	X	X
Aorta		X	X
Bone marrow (sternum) [a]		X	X
Brain (cerebrum, cerebellum, medulla/pons evaluated)	X	X	X
Cecum		X	X
Cervix		X	X
Clitoral gland		X	
Colon		X	X
Duodenum		X	X
Epididymis (2)	X	X	X
Esophagus		X	X
Exorbital/lacrimal gland		X	
Eye (2; preserved in Davidson's fixative)		X	X
Fallopian tube (oviduct) (2)		X	X
Femur (articular surface of distal end)		X	X
Gross lesions		X	X
Harderian gland (2; preserved in Davidson's fixative)		X	X
Heart	X	X	X
Ileum (include Peyer's patch)		X	X
Jejunum		X	X
Kidney (2)	X	X	X
Larynx		X	X
Liver	X	X	X
Lung with large bronchi		X	X
Lymph node (mandibular) (2)		X	X
Lymph node (mesenteric)		X	X
Mammary gland (both sexes)		X	X
Muscle (gastrocnemius)		X	X
Nasopharynx		X	X
Nasal structure		X	X
Optic nerve (2; preserved in Davidson's fixative)		X	X
Ovary (2)	X	X	X
Pancreas		X	X

Tissue	Organ Weight Taken	Collected and Preserved	Microscopic Examination
Parathyroid gland[b]		X	
Pituitary gland		X	X
Physical identifier[c]		X	
Preputial gland		X	
Prostate		X	X
Rectum		X	X
Salivary gland [mandibular (2)]		X	X
Seminal vesicle (with coagulating gland) (2)	X	X	X
Sciatic nerve		X	X
Skin/subcutis (treated)		X	X
Skin (untreated)		X	X
Spinal cord (cervical, thoracic, and lumbar)		X	X
Spleen	X	X	X
Stomach		X	X
Testis (2; preserved in modified Davidson's fixative)	X	X	X
Thymus	X	X	X
Thyroid with parathyroid (2)		X	X
Tongue		X	X
Trachea		X	X
Urinary bladder		X	X
Uterus	X	X	X
Vagina		X	
Zymbal's gland		X	

[a] Entered in DATATOX as bone marrow.

[b] Parathyroid gland was collected with the thyroid gland.

[c] Collected and maintained with preserved tissues.

D. Results

a. Mortality and clinical observations

Survival was unaffected by administration of the test substance. All animals survived to their scheduled sacrifice.

Clinical signs of toxicity attributable to the test substance were not observed in this study.

b. Body weight

Body weights and body weight gain, presented in Table 5.3.7-03, were measured weekly were not affected by test substance administration.

Table 5.3.7-03: Mean Body Weight Gain and Body Weights (g) in Male and Female Rats during the 28-Day Study with BYI 02960

Dosage Level of BYI 02980	0 mg/kg	50 mg/kg	150 mg/kg	500 mg/kg
Males				
Initial BW (Day 0) (%C)	264	265 (100%)	265 (100%)	265 (100%)
BWG Week 1 (Days 0–7)	**16**	**18**	**15**	**20**
BWG Weeks 1–4 (Days 0–28)	**59**	**67**	**59**	**71**
Final BW (Day 28) (%C)	323	331 (102%)	324 (100%)	336 (104%)
Females				
Initial BW (Day 0) (%C)	178	177 (99%)	178 (100%)	176 (98%)
BWG Week 1 (Days 0–7)	**14**	**10**	**15**	**13**
BWG Weeks 1–4 (Days 0–28)	**40**	**38**	**40**	**39**
Final BW (Day 28) (%C)	218	215 (97%)	218 (100%)	215 (98%)

c. Food consumption

Food consumption was unaffected in males at all dose levels tested.

In the first two weeks of daily dermal dosing, 500 mg/kg/day females had significantly lower food consumption (g/animal/day) as compared to controls. In addition, food efficiency (g/kg bw/day) was significantly lower in the first week. Females dosed with 50 mg/kg/day had significantly lower food consumption in the second week as compared to controls. These findings were not considered to be biologically significant as changes were not consistently affected in a dose-related fashion, were not found in males, and did not impact clinical observations or body weights at any point. The data are presented in Table 5.3.7-04.

Table 5.3.7-04: Mean Food Consumption (g/Day) in Male and Female Rats during the 28 Day Study with BYI 20960

Dosage Level of BYI 02960	**0 mg/kg**	**50 mg/kg**	**150 mg/kg**	**500 mg/kg**
Males				
Week 1 (Days 0–7) (%C)	**20.87**	**21.14 (10%)**	**20.77 (100%)**	**20.91 (100%)**
Weeks 1–4 (Days 0–28) (%C)	21.8	22.7 (104%)	21.9 (100%)	22.6 (104%)
Females				
Week 1 (Days 0–7) (%C)	**17.11**	**15.78 (92%)**	**16.64 (97%)**	**15.12 (88%)**
Weeks 1–4 (Days 0–28) (%C)	18.6	17.1 (92%)	18.0 (96%)	17.1 (92%)

d. Ophthalmologic examinations

Ophthalmologic changes attributable to the test substances were not observed in this study.

e. Hematology and clinical chemistry

Hematology or coagulation parameter changes attributable to the test substance were not observed in this study.

Clinical chemistry parameter changes attributable to the test substance were not observed in this study.

f. Sacrifice and pathology

1. Organ weights
Test substance-related organ weight changes were noted in liver at 500 mg/kg/day males.

The absolute and relative liver weights at 500 mg/kg/day males were decreased at the rate of 6% and 9%, respectively, as compared to control rats. However, there were no test substance-related clinical or gross or microscopic pathology changes to correlate this finding.

There were no other test substance-related organ weight changes.

2. Gross Pathology
There were no test substance-related gross lesions observed in either sex.

3. Micropathology
There was no test substance-related micropathology change in males or females at any dose level tested.

Conclusions

Based on the lack of adverse findings observed in this study, the 28-day dermal exposure NOAEL in male and female rats dosed with BYI 02960 was 500 mg/kg/day.

KIIA 5.3.8 - Percutaneous 90-day toxicity (rodents)

Based on the results of the acute and 28-day dermal toxicity studies and the physical properties of BYI 02960, a 90-day repeat dermal study is not triggered in the US or EU.

KIIA 5.4 - Genotoxicity

BYI 02960 was tested in a standard battery of *in vitro* and *in vivo* genotoxicity studies and mutagenicity tests *in vitro* and *in vivo* carried out according to the current OECD and European guidelines. The studies were performed between 2009 and 2011 in compliance with GLP requirements. There was no indication of gene mutation in either the presence or absence of metabolic activation in both the bacterial reverse mutation and mammalian gene mutation tests. The *in vitro* chromosome aberration test and the *in vivo* mouse micronucleus tests were also both negative. These studies demonstrate that BYI 02960 has no genotoxic potential.

Table 5.4-01: Summary of genotoxicity test

Mutagenicity tests with BYI 02960	Metabolic Activation	Results
A. *In vitro* tests		
Ames Test (M-354173-01-2)	+/-	Negative
Ames Test (M-420539-02-2)	+/-	Negative
Chromosome aberrations (V79 cells) (M-359746-01-2)	+/-	Negative
HPRT Test (V79 cells) (M-359743-01-2)	+/-	Negative
B. *In vivo* tests	**Dose levels**	
Micronucleus Test in male mice – oral administration (M-353785-01-2)	10, 20 and 40 mg/kg	Negative
Micronucleus Test in female mice – oral administration (M-420536-01-2)	12.5, 25 and 50 mg/kg	Negative

KIIA 5.4.1 - *In vitro* genotoxicity - Bacterial assay for gene mutation

Report:	**KIIA 5.4.1/01, [redacted] B.; 2009**
Title:	BYI 02960, Salmonella/microsome test, plate incorporation and preincubation method
Report No & Document No	AT05387 M-354173-01-2
Guidelines:	OECD 471 (1997); EEC Directive 2000/32/EC Method B13/14 (2000); EPA Health Effects Test Guideline (OPPTS 870.5100, 1998)
GLP	Yes (certified laboratory)

Executive Summary

In this *in vitro* assessment of the mutagenic potential of BYI 02960 (Batch 2009-000239, 96.2% of purity), histidine dependent auxotrophic mutants of Salmonella typhimurium, strains TA 1535, TA 1537, TA 98, TA 100 and TA 102 were exposed to BYI 02960 diluted in dimethyl sulphoxide (DMSO) at concentrations up to 5000 µg/plate,. For each bacterial strain and dose level, triplicate plates were used in both the presence and absence of an Aroclor 1254-induced rat liver metabolic activation system (S9 mix). DMSO was also used as a negative control. Specific positive controls were used for each strain. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were scored using an automated colony counter. Another assay testing a pre-incubation for 20 minutes at 37 °C was also performed at doses ranging from 16 to 5000 µg/plate.

At 5000 µg per plate, the substance had a weak, strain-specific bacteriotoxic effect. Due to the weakness of this effect this dose could nevertheless be used for assessment purposes.

BYI 02960 did not cause any significant increase in the number of revertant colonies in either the presence or absence of metabolic activation.

All the positive control compounds produced expected increases in the number of revertant colonies, thereby demonstrating the sensitivity of the assay and the efficacy of the S9 mix.

Therefore, BYI 02960 was non-mutagenic with or without S9 mix in the plate incorporation as well as in the pre-incubation modification of the Salmonella/microsome test.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960

Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable for the duration of the study

2. Control materials:

Negative:	Culture medium
Solvent:	DMSO
Positive:	Sodium azide (Fluka) for TA 1535 at 10 + 20 µg/plate, Nitrofurantoin (Sigma) for TA 100 at 0.2 + 0.4 µg/plate, 4-Nitro-1,2-phenylene diamine (Merck-Schuchardt) for TA 1537 at 10 + 20 µg/plate and TA 98 at 0.5 + 1 µg/plate, Mitomycin C (Fluka) for TA 102 at 0.2 + 0.4 µg/plate only in plate incorporation plate, Cumene hydroperoxide (Sigma) for TA 102 in pre-incubation trials only at 50 + 75 µg/plate, 2-Aminoanthracene (Fluka) for the activating effect of the S9 mix in all strains at 3 + 6 µg/plate

3. Test organisms:

Species:	Salmonella typhimurium LT2 mutants
Strain:	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102
Source:	Strains obtained from [redacted] in 1997 and stored in the laboratory since then

4. Test compound concentrations:

Plate incorporation assay: First assay for all strains with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/plate

Pre-incubation assay: For TA 1535, TA 1537, TA98, TA100 and TA 102 with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/tube

B. Study Design and methods

The experimental phase of the study was performed between June 17 to 29, 2009 at [redacted]

The Salmonella/microsome test is an *in vitro* screening method which detects point mutations caused by chemical agents. Auxotrophic mutants of Salmonella typhimurium are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

1. Plate incorporation assay

BYI 02960 or the control material were dissolved in 0.1 mL of DMSO. DMSO (0.1 mL) containing BYI 02960 or controls were added to glass vessels with 0.1 mL of bacterial cultures grown overnight, 0.5 mL of S9 mix or buffer and 2 mL of soft agar. The mixture was placed in a water bath at 45 °C for 30 seconds, shaken and overlaid onto Petri dishes containing solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were scored using an automated colony counter. Three plates were used, both with and without S9 mix, for each strain and dose. The doses for the first trial were routinely determined on the basis of a standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses. If less than three doses were used for assessment, at least two repeats were performed.

2. Pre-incubation assay

An independent repeat was performed as pre-incubation of the previously described mixture in a water bath at 37 °C for 20 minutes. At the end of the preincubation period, 2 mL of molten soft agar were added to the tubes, the content mixed and plated onto Petri dishes with solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were also scored using an automated colony counter.

3. Assessment criteria

A reproducible and dose-related increase in mutant colonies of at least one strain was considered to be positive. For TA 1535, TA 100 and TA 98, this increase should be about twice that of negative controls, whereas for TA 1537, at least a threefold increase should be reached. For TA 102 an increase of about 100 mutants should be reached. Otherwise, the result was considered as negative.

H. Results and discussion

There was no indication of a bacteriotoxic effect of BYI 02960 at doses of up to and including 1581 µg per plate. The total bacteria counts consistently produced results comparable to the negative controls, or differed only insignificantly. No inhibition of growth was noted as well. 5000 µg per plate had only a weak, strain-specific bacteriotoxic effect. Therefore this dose could nevertheless be used for assessment purposes.

None of the five strains concerned showed in the plate incorporation test a dose related and biologically relevant increase in mutant counts over those of the negative controls. This applied both to the tests with and without S9 mix and was confirmed by the results of the preincubation trials.
The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene increased mutant counts to well over those of the negative controls, and thus demonstrated the system's sensitivity and the activity of the S9 mix.

Table 5.4-02: Mean mutant values per plate in the plate incorporation assay

Test item	Concentration µg/plate	S9 mix	Strains				
			TA 1335	TA 100	TA 1537	TA 98	TA 102
BYI 02960	0	-	8	124	7	25	212
	16	-	8	128	6	26	216
	50	-	7	121	6	27	218
	158	-	7	145	5	24	207
	500	-	7	135	6	24	212
	1581	-	8	131	5	28	220
	5000	-	7	116	9	24	223
Na-azide	10	-	805				
	20	-	835				
NF	0.2	-		341			
	0.4	-		406			
4-NPDA	10	-			55		
	20	-			52		
	0.5	-				68	
	1	-				117	
MMC	0.2	-					657
	0.4	-					742
BYI 02960	0	+	12	198	10	31	294
	16	+	10	182	8	38	297
	50	+	10	190	10	25	314
	158	+	10	177	8	35	335
	500	+	11	213	8	29	314
	1581	+	8	202	7	26	288
	5000	+	7	188	7	35	214
2-AA	3	+	90	1719	304	2031	613
	6	+	59	1881	135	2221	1183

Table 5.4-03: Mean mutant values per plate in the pre-incubation assay

Test item	Concentration µg/plate	S9 mix	Strains				
			TA 1335	TA 100	TA 1537	TA 98	TA 102
BYI 02960	0	-	8	128	7	17	220
	16	-	9	113	7	19	197
	50	-	8	112	6	17	215
	158	-	8	126	7	19	227
	500	-	7	131		19	199
	1581	-	9	116	6	18	197
	5000	-	8	107	7	18	173
Na-azide	10	-	629				
	20	-	746				
NF	0.2	-		412			
	0.4	-		638			
4-NPDA	10	-			32		
	20	-			71		
	0.5	-				23	
	1	-				154	
Cumene	50	-					418
	75	-					448
BYI 02960	0	+	10	156	10	35	263
	16	+	11	164	8	31	224
	50	+	9	150	10	33	248
	158	+	9	190	7	32	268
	500	+	10	183	9	33	311
	1581	+	9	165	8	22	245
	5000	+	8	116	9	28	244
2-AA	3	+	82	1405	309	917	553
	9	+	60	1914	239	1065	1191

III. Conclusions

No indication of mutagenic effects of BYI 02960 could be found at assessable doses of up to 5000 µg/plate in any of the Salmonella typhimurium strains used in the assay.

Report:	**KIIA 5.4.1/02, [redacted] A.; 2011**
Title:	Salmonella Typhimurium, Reverse mutation assay with BYI 02960
Report No & Document No	1425802 M-420539-02-2
Guidelines:	OECD 471 (1997); EEC Directive N° 440/2008 B13/14 (2008); EPA Health Effects Test Guideline (OPPTS 870.5100; 1998)
GLP	Yes (certified laboratory) except that no analytical analyses were performed during the study

Executive Summary

Based on the specification required for production of the technical BYI 02960, a new Ames test was performed. In this *in vitro* assessment of the mutagenic potential of BYI 02960 (Batch PFV107N005, 97.2% of purity), histidine dependent auxotrophic mutants of Salmonella typhimurium, strains TA 1535, TA 1537, TA 98, TA 100 and TA 102 were exposed to BYI 02960 diluted in dimethyl sulphoxide (DMSO) at concentrations up to 5000 µg/plate. For each bacterial strain and dose level, triplicate plates were used in both the presence and absence of an Aroclor 1254-induced rat liver metabolic activation system (S9 mix). DMSO was also used as a negative control. Specific positive controls were used for each strain. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were scored using an automated colony counter. Another assay testing a pre-incubation for 60 minutes at 37 °C was also performed at doses from 33 to 5000 µg/plate.

At 5000 µg per plate in experiment I, a minor reduction in the number of revertants (below the indication factor of 0.5) was observed in strain 1537 with and without S9 mix.

BYI 02960 did not cause any significant increase in the number of revertant colonies in either the presence or absence of metabolic activation.

All the positive control compounds produced expected increases in the number of revertant colonies, thereby demonstrating the sensitivity of the assay and the efficacy of the S9 mix.

Therefore, BYI 02960 was non-mutagenic with or without S9 mix in Salmonella Typhimurium reverse mutation assay.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960

Description:	Beige powder
Lot/Batch:	PFV107N005
Purity:	97.2%
CAS:	951659-40-8
Stability of test compound:	No analysis performed during the study

2. Control materials:

Negative:	Culture medium
Solvent:	DMSO
Positive:	Sodium azide (Serva) for TA 1535 and TA 100 at 10 µg/plate, 4-Nitro-1,2-phenylene diamine (Fluka) for TA 1537 at 50 µg/plate and TA 98 at 10 µg/plate, Methyl methane sulfonate (Sigma Aldrich) for TA 102 at 3.0 µL/plate, 2-Aminoanthracene (Sigma Aldrich) for the activating effect of the S9 mix in all strains at 2.5 µg/plate for all strains except TA 102 at 10.0 µg/plate

3. Test organisms:

Species:	Salmonella typhimurium LT2 mutants
Strain:	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102
Source:	[redacted] Germany)

4. Test compound concentrations:

Experiment I:	First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Experiment II:	For TA 1535, TA 1537, TA98, TA100 and TA 102 with or without S9 mix: 33, 100, 333, 1000, 2500 and 5000 µg/tube

B. Study Design and methods

The experimental phase of the study was performed between August 08 to 23, 2011 at [redacted], Germany.

The Salmonella/microsome test is an *in vitro* screening method which detects point mutations caused by chemical agents. Auxotrophic mutants of Salmonella typhimurium are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

1. Plate incorporation assay (experiment I)

DMSO (0.1 mL) containing BYI 02960 or controls were added to glass vessels containing 0.1 mL of bacterial cultures grown overnight, 0.5 mL of S9 mix or buffer and 2 mL of soft agar. The mixture was placed in a water bath at 45 °C for 30 seconds, shaken and overlaid onto Petri dishes containing solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were scored using the Petri Viewer Mk2. Due to air bubbles, the colonies were partly counted manually. Three plates were used, both with and without S9 mix, for each strain and dose. The doses for the first trial were routinely determined on the basis of a standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses. If less than three doses were used for assessment, at least two repeats were performed.

2. Pre-incubation assay (experiment II)

An independent repeat was performed as pre-incubation of the previously described mixture in a water bath at 37 °C for 60 minutes. At the end of the preincubation period, 2 mL of molten soft agar were added to the tubes, the content mixed and plated onto Petri dishes with solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were also scored using an automated colony counter.

3. Assessment criteria

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100 and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed. A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration. An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and discussion

The plates incubated with the test item showed normal background growth up to test item levels of 5000 µg/plate with or without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with or without metabolic activation. Only in experiment I in strain TA 1537 at 5000 µg/plate was a minor reduction in the number of revertants (below the indication factor of 0.5) observed both with and without S9 mix.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BYI 02960 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance. Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

Table 5.4-04: Mean mutant values per plate in the plate incorporation assay

Test item	Concentration µg/plate	S9 mix	Strains				
			TA 1335	TA 1537	TA 98	TA 100	TA 102
DMSO			16	13	29	139	491
Untreated			20	17	48	173	485
BYI 02960	3	-	13	12	30	136	493
	10	-	17	14	30	141	488
	33	-	18	11	31	134	434
	100	-	16	12	32	147	452
	333	-	16	12	32	156	462
	1000	-	18	8	28	140	458
	2500	-	13	11	31	149	450
	5000		14	4	35	132	390
Na-azide	10	-	2100			2135	
4-NOPD	10	-			275		
	50			63			
MMS	3 µL	-					4489
DMSO			20	13	39	174	633
Untreated			17	24	37	162	540
BYI 02960	3	+	19	16	40	175	610
	10	+	19	13	36	152	611
	33	+	21	14	40	159	612
	100	+	21	15	39	163	649
	333	+	20	15	38	164	622
	1000	+	20	15	39	172	629
	2500	+	19	14	44	153	636
	5000	+	18	5	36	160	543
2-AA	2.5	+	407	344	2932	2911	
	10	+					2782

Table 5.4-05: Mean mutant values per plate in the pre-incubation assay

Test item	Concentration µg/plate	S9 mix	Strains				
			TA 1335	TA 1537	TA 98	TA 100	TA 102
DMSO			12	18	29	120	383
Untreated			12	16	47	136	338
BYI 02960	33	-	13	15	29	132	354
	100	-	12	19	30	106	373
	333	-	13	16	27	128	380
	1000	-	15	18	24	96	330
	2500	-	14	17	31	96	321
	5000		15	19	26	89	283
Na-azide	10	-	1837			1793	
4-NOPD	10	-			303		
	50	-		93			
MMS	3 µL	-					1084
DMSO			20	20	40	122	366
Untreated			15	18	42	157	356
BYI 02960	33	+	24	18	42	115	433
	100	+	20	20	37	122	385
	333	+	20	22	47	122	449
	1000	+	20	17	44	109	487
	2500	+	18	15	34	118	411
	5000	+	17	19	37	106	278
2-AA	2.5	+	264	246	1612	2218	
	10	+					1897

III. Conclusions

No indication of mutagenic effects of BYI 02960 could be found at assessable doses of up to 5000 µg/plate in any of the Salmonella typhimurium strains used in the assay.

KIIA 5.4.2 - *In vitro* genotoxicity - Test for clastogenicity in mammalian cells

Report:	**KIIA 5.4.2/01, [redacted] M.; 2009**
Title:	BYI 02960, *In vitro* chromosome aberration test with Chinese Hamster V79 cells
Report No & Document No	AT05626 M-359746-01-2
Guidelines:	OECD 473 (1997); EEC Directive 2000/32/EC Method B10 (2000); EPA Health Effects Test Guideline (OPPTS 870.5375; 1998)
GLP	Yes (certified laboratory)

Executive Summary

In this *in vitro* assessment of the clastogenic potential of BYI 02960 (batch 2009-000239, 96.2% of purity), Chinese Hamster V79 cells were exposed to BYI 02960 diluted in dimethyl sulphoxide (DMSO) at 500, 1000, 2000, 2500 and 3000 µg/mL. For each dose level, duplicate cultures were used in both the presence and absence of an Aroclor 1254-induced rat liver metabolic activation system (S9 mix). DMSO was also used as a negative control. Mitomycin C, which produces crosslinks in the DNA, and cyclophosphamide, which induces chromosomal damage after metabolic activation, were used as positive controls, diluted in Hanks' balanced salt solution. After 4 hours treatment, the medium was changed and the cells were harvested 14 hours later. A second harvest was performed 30 hours after the start of the study for the cells treated with 2000, 2500 and 3000 µg/mL. An additional experiment at BYI 02960-concentrations of 100, 200, 400, 600 and 800 µg/mL without S9 was performed using continuous treatment for 18 hours with harvest at the same time. Colcemid was added to each flask two hours prior to harvest to arrest the cells in a metaphase-like stage of mitosis.

Without S9 mix cytotoxic effects were observed at 1000 µg/mL and above after 4 hours treatment and at 400 µg/mL and above after 18 hours treatment. With S9 mix cytotoxic effects were observed at 2000 µg/mL and above. Precipitation in the medium did not occur. Therefore, concentrations of 500, 1000 and 2500 µg/mL BYI 02960 (4 hours treatment) and 200, 400 and 800 µg/mL (18 hours treatment) were chosen for reading in the absence of S9 mix. In the presence of S9 mix 500, 1000 and 3000 µg/mL of BYI 02960 were employed. All of these cultures harvested 18 hours after the beginning of the treatment were included. In addition, cultures treated in the absence of S9 mix with 2500 µg/mL and harvested 30 hours after the beginning of the treatment were used. The same was true for cultures treated in the presence of S9 mix with 3000 µg/mL.

None of the cultures treated with BYI 02960 showed biologically relevant or statistically significant increased numbers of aberrant metaphases either in the absence or in the presence of S9 mix.

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.

BYI 02960 was considered not to be clastogenic for mammalian cells *in vitro*.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	Batch 2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable for the duration of the study
2. Control materials:	
Negative:	Culture medium
Solvent:	DMSO for BYI 02960 and Hanks's balanced salt solution for positive controls
Positive:	Mitomycin C (Fluka, Biochemika) without S9 mix at 0.1 µg/mL for a treatment period of 4 hours, and 0.03 µg/mL for a treatment period of 18 hours Cyclophosphamide (Endoxan 100 mg injection vials of dry substance, Baxter Oncology GmbH) with S9 mix at 2 µg/mL
3. Test organisms:	
Cell line:	Chinese hamster V79 lung cells
Source:	Cells obtained from [redacted] in 1993, stored in the laboratory since then
4. Test compound concentrations:	BYI 02960 was used in trials without S9 mix at 500, 1000 and 2500 µg/mL and in trials with S9 mix at 500, 1000 and 3000 µg/mL

B. Study Design and methods

The experimental phase of the study was performed from May 26 to October 5, 2009 at [redacted]

The *in vitro* cytogenetic test is a mutagenicity test system for the detection of chromosome aberrations in cultured mammalian cells. The test is designed to detect structural aberrations (chromatid and chromosome aberrations) in cells at their first post-treatment mitosis.

1. Determination of cytotoxicity

In a pre-test, duplicate cultures were exposed to BYI 02960 at concentrations ranging from 1 to 3000 µg/mL with or without S9 mix for 4 hours. In addition, cells were exposed without S9 mix for 18 hours to concentrations ranging from 10 to 3000 µg/mL. The mitotic index was determined for all cultures. The number of mitotic cells among a total of 1000 cells per culture was determined. All cells which were not in interphase were defined as mitotic.

In the main study, cultures with a total incubation period of 8 hours were additionally and exclusively used to determine the cytotoxicity of BYI 02960 at concentrations ranging from 500 to 3000 µg/mL.

Concentrations of up to 3000 µg/mL BYI 02960 did not change the pH or the osmolality in the medium of the pre-test.

2. Treatment protocol

Approximately 1x10^6 cells were seeded in 20 mL of medium per 75 cm^2 flasks and incubated at 37 °C in a CO_2-incubator (5% CO_2). Unless reported otherwise, the cells were grown in Eagle's minimal essential medium containing 10% foetal calf serum. Immediately before treatment, the medium was removed from the cultures. For the trials without S9 mix, 20 mL of medium containing 2% foetal calf serum and 0.2 mL of test substance solution were added to each flask. For the trials with S9 mix, 19 mL of medium containing 2% foetal calf serum, 1 mL of S9 mix and 0.2 mL of test substance solution were added to each flask. The cells were incubated for 4 hours at 37 °C. After 4 hours of treatment, the medium was removed, the cells were washed with PBS and 20 mL of fresh medium containing 10% foetal calf serum was added to the flasks. The flasks were placed in a CO_2-incubator for the remaining incubation time. In the cultures treated for 18 hours, the medium was not removed.

0.2 mL of Colcemid-solution (40 µg/mL) was added to each flask 2 hours prior to the end of the incubation period to arrest the cells in a metaphase-like stage of mitosis(c-metaphase).

Positive controls and solvent controls (0.2 mL of solvent per culture) were set up in parallel and handled as described for BYI 02960-treated cultures. Untreated controls and solvent controls were used as negative controls.

3. Chromosome preparations

After the removal of the medium from each flask, the cells were trypsinized, suspended in medium and centrifuged for approximately 5 minutes at 700 rpm. The supernatant was removed and 1 to 2 mL of a hypotonic solution (0.4% KCl; 37 °C) was added to each tube. Within 4 minutes, the volume was brought to 6 mL with additional hypotonic solution and cells were resuspended. The cells were centrifuged again and fixed with cold (4 °C) fixative (ethanol/acetic acid 3:1) for 20 minutes at room temperature. Cells were pelleted and resuspended in fixative as before and centrifuged again. The pelleted cells were resuspended in a small volume of fixative and the suspension was dropped onto clean slides. The slides were allowed to dry for at least 2 hours. Thereafter, they were submerged in pure methanol for 3 minutes and stained for 15-20 minutes in 3% Giemsa solution. Slides were rinsed twice in water and once in acetone and were then kept in xylene for about 30 minutes. The slides were allowed to dry completely and covered. At least two slides were generated per culture.

4. Evaluation criteria

Coded slides were evaluated using a light microscope at a magnification of about 1000. Chromosomes of approximately 200 metaphases per concentration, 100 metaphases from each of two parallel cultures, were examined. Only metaphases containing the modal chromosome number (22) were analyzed unless exchanges were detected. The following aberrations were recorded: gaps (an achromatic lesion within a chromatid arm without dislocation of the chromatid end), break (a discontinuity of one chromatid with dislocation of the chromatid end), fragment (part of chromosome without centromere), deletion (result of a break with the terminal chromatid part of the chromosome missing within the metaphase under assessment), exchange (exchange of chromatid-parts between different chromosomes or within the

same chromosome), multiple aberration (when five or more structural changes occur within one metaphase). Observed polyploidy metaphases were recorded but not used for assessment.

5. Assessment criteria

An assay was acceptable if there was a biologically relevant increase in chromosome aberrations induced by positive controls and if the numbers of aberrations for the negative controls were in the expected range.
An increased incidence of gaps of both types without a concomitant increase of other aberration types was considered not to be an indication of a clastogenic effect.
A test was considered positive if there was a relevant and statistically significant increase in the aberration rate.
A test was considered negative if there was no such increase at any time interval or if there were statistically significant values, which were, however, within the range of historical negative controls.
A test was considered equivocal if there was an increase above the range of historical negative controls which was statistically significant but not considered relevant, or if an increase occurred, which was considered relevant, but which was not statistically significant.

6. Statistics

The statistical analysis was performed by pair-wise comparison of BYI 02960-treated and positive control groups to the respective solvent control group.
The mitotic index was statistically analyzed (provided that it was reduced compared to the mean of the corresponding solvent control) using the one-sided Chi^2-test.
The numbers of metaphases with aberrations excluding gaps were compared (provided that these data superceded the respective solvent control). The one-side Chi^2-test was used for the statistical evaluation.
A difference was considered to be significant, if the probability of error was below 5%.

II. Results and discussion

1. Survival Index

Survival index without S9 mix

In comparison to the solvent control, the survival indices in the treated cultures were relevantly reduced at 1000 µg/mL and above (4 hours treatment) and at 600 µg/mL and above (18 hours treatment). The cultures treated with mitomycin C also showed a reduction in survival rate.

Survival index with S9 mix

In comparison to the solvent control, the treated cultures showed a relevant reduction in the survival rate at 2000 µg/mL and above. The positive control cyclophosphamide also reduced the survival rate.

2. Mitotic Index

Mitotic index without S9 mix

In comparison to the solvent control, the mitotic indices in the treated cultures were relevantly reduced at 2000 µg/mL and above (4 hours treatment) and at 400 µg/mL and above (18 hours treatment). The cultures treated with mitomycin C showed a reduction in mitosis rate only at the 4 hours treatment.

Mitotic index with S9 mix

In comparison to the solvent control, the treated cultures showed a relevant reduction in the mitosis rate at 2000 µg/mL and above. The positive control cyclophosphamide also reduced the mitosis rate.

3. Chromosome aberrations

BYI 02960 without S9 mix

No biologically relevant or statistically significant increases of numbers of metaphases with aberrations were detected after 4 hours treatment and total culture times of 18 or 30 hours. The same was true for a treatment period and total culture time of 18 hours. The treatment with the positive control mitomycin C resulted in a clear and statistically significant increase of metaphases with aberrations and demonstrated the sensitivity of the test system.

BYI 02960 with S9 mix

No biologically relevant or statistically significant increases of numbers of metaphases with aberrations were detected after 4 hours treatment and total culture times of 18 or 30 hours. The positive control cyclophosphamide induced statistically significant and biologically relevant increases of metaphases with aberrations and demonstrated the sensitivity of the test system and the activity of the used S9 mix.

Table 5.4-06: Mean numbers of aberrant cells

Test items	Concentration µg/mL	Harvest time (hours)	S9 mix	Aberrant cells in %	
				Including gaps	Excluding gaps
4 hours treatment					
DMSO		18	-	1.0	1.0
BYI 02960	500	18	-	4.0	3.5
	1000	18	-	1.5	1.5
	2500	18	-	4.0	4.0
Mitomycin C	0.1	18	-	56.5	55.5**
DMSO		18	+	3.5	2.0
BYI 02960	500	18	+	2.5	2.0
	1000	18	+	3.0	1.5
	3000	18	+	3.5	3.5
Cyclophosphamide	2	18	+	74.0	71.5**
DMSO		30	-	1.5	1.0
BYI 02960	2500	30	-	2.0	2.0
DMSO		30	+	3.0	1.5
BYI 02960	3000	30	+	3.0	3.0
18 hours treatment					
DMSO		18	-	3.0	3.0
BYI 02960	200	18	-	4.0	4.0
	400	18	-	2.5	2.0
	800	18	-	3.5	3.5
Mitomycin C	0.03	18	-	52.0	51.5**

** : $p \leq 0.01$

III. Conclusions

None of the cultures treated with BYI 02960 in the absence or presence of S9 mix showed statistically significant or biologically relevant increases of numbers of metaphases with aberrations.

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

Based on the results of this test, BYI 02960 is considered not to be clastogenic for mammalian cells *in vitro*.

KIIA 5.4.3 - *In vitro* genotoxicity - Test for gene mutation in mammalian cells

Report:	**KIIA 5.4.3/01, [redacted] G.; 2009**
Title:	BYI 02960, V79/HPRT-test *in vitro* for the detection of induced forward mutations
Report No & Document No	AT05625 M-359743-01-2
Guidelines:	OECD 476 (1997); EEC Directive 2000/32/EC Method B17 (2000); EPA Health Effects Test Guideline (OPPTS 870.5300; 1998)
GLP	Yes (certified laboratory)

Executive Summary

The purpose of the study was to assess the point mutagenic potential of BYI 02960 (batch 2009-000239, 96.2 % of purity) at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus in V79 cells.

For dose selection, a preliminary cytotoxicity test was conducted with and without an Aroclor 1254-induced rat liver metabolic activation system (S9 mix) using concentrations of BYI 02960 ranging from 15.6 to 3000 µg/mL. No cytotoxic effects were observed. BYI02960 was tested in the first mutation experiment and the following independent repeats at concentrations ranging from 46 to 2944 µg/mL.

Exponentially growing V79 cells were plated in 20 mL culture medium in two 75 cm^2 flasks. After attachment, cells were exposed with or without S9 mix to vehicle alone or to a range of concentrations of the test substance for 5 hours. Thereafter, each cell monolayer was washed with PBS, trypsinized and replated into 3 Petri dishes. One flask and 3 Petri dishes were used per culture. These dishes were incubated for 6 to 8 days to allow colony development and to determine the cytotoxicity associated with each test substance directly after treatment (survival to treatment).

Cells in 75 cm^2 flasks were incubated to permit growth and expression of induced mutations. Cells were subcultured (= count 1, normally after 3 days) by reseeding cells in 75 cm^2 flasks. At the end of the expression period (= count 2, normally a total of 6 days), cultures were reseeded in Petri dishes without hypoxanthine but containing 6-TG for selection of mutants. In addition, 200 cells per dish were seeded in 5 mL culture medium to determine the absolute cloning efficiency for each concentration. After incubation for 6 to 8 days, the colonies were fixed, stained with Giemsa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.

BYI 02960 was tested up to 2944 µg/mL, a concentration which slightly exceeded the limit concentration of 10mM. Precipitation of BYI 02960 in the culture medium was observed at 2944 µg/mL. BYI 02960 induced no decreases in survival to treatment or in relative population growth either without or with S9 mix. There was no biologically relevant increase in mutant frequency above that of the negative controls either without or with S9 mix.

The positive controls ethyl methanesulfonate and dimethylbenzanthracene induced clear mutagenic effects and demonstrated the sensitivity of the test system and the activity of the S9 mix.

Based on these results, BYI 02960 is considered to be non-mutagenic in the V79/HPRT Forward Mutation Assay, both with and without metabolic activation.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Fine crystalline brownish powder
Lot/Batch: Batch 2009-000239
Purity: 96.2%
CAS: 951659-40-8
Stability of test compound: Stable for the duration of the study

2. Control materials:
Negative: Culture medium [Eagle's minimal essential medium supplemented with 1% L-glutamine, 1% MEM-vitamins, 1% MEM NEAA, 1% penicillin/streptomycin and 10% foetal calf serum (FCS)]
Solvent: DMSO for BYI 02960 and Dimethylbenzanthracene not exceeding 1% (v/v) in the culture medium. No solvent needed for ethyl methanesulfonate as it is a liquid
Positive: Ethyl methanesulfonate (EMS), a directly alkylating agent, used at a final concentration of 900 µg/mL in non-activation trials.
Dimethylbenzanthracene (DMBA), promutagen requiring a metabolic activation, used at a final concentration of 20 µg/mL for trials with S9 mix.

3. Test organisms:
Cell line: Chinese hamster V79 lung cells
Source: Cells obtained from [redacted], in 2008. These cells have since been reclomed to maintain karyotypic stability. They have a modal chromosome number of 22 and a rapid population doubling time (10 to 14 hours).
Culture condition: Incubation performed at 37°C in a humidified atmosphere with about 5% CO_2

4. Test compound concentrations:
BYI 02960 was used at 15.6, 31.3, 62.5, 125, 250, 500, 1000, 2000 and 3000 µg/mL in the clonal cytotoxicity assay and at 46, 92, 184, 368, 736, 1472 and 2944 µg/mL in the mutagenic assays

5. Metabolic activation:

The S9 fraction was isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats. The preparation dated from January 26, 2009 (protein content from 24.5 mg/mL) and was kept frozen at -80 °C. The batch was tested for contamination, cytotoxicity and its metabolizing capacity by using 20 µg/mL DMBA prior to use in the first study. Cofactors were freshly dissolved in sodium phosphate buffer (150 mM, pH 7.4).

B. Study Design and methods

The experimental phase of the study was performed April 21 to September 25, 2009 at [redacted]

The selection of V79 forward mutations is based on the resistance of induced mutants to the purine analogue 6-thioguanine (6-TG). This resistance is a result of a mutation at the X-chromosome-linked HPRT locus rendering the cells unable to use 6-TG for DNA synthesis. Therefore, cell colonies formed in the presence of 6-TG are considered to represent mutants at the HPRT gene.

1. Determination of cytotoxicity

Exponentially growing V79 cells were plated in 20 mL culture medium in a 75 cm^2 flask with a total volume of 275 mL ($4x10^6$ cells per flask). For each concentration, one culture was available. After attachment (16 to 24 hours later), cells were exposed without S9 mix to vehicle alone or to a range of concentrations of the test substance for 5 hours in 20 mL medium containing 2% FCS. In experiments with metabolic activation 1 mL of medium was replaced by 1 mL of S9 mix. Thereafter, each cell monolayer was washed with PBS, trypsinized and replated in 5 mL culture medium at a density of 200 cells into 3 Petri dishes (diameter of 60 mm). These dishes were incubated for 6 to 8 days to allow colony development. Thereafter, colonies were fixed with 95% methanol, stained with Giemsa (Merk; stock solution diluted 1:5 with deionized water) and counted automatically using Biologics Accu Count 1000, when there was no interference by precipitation on the plates or coloration of the plates. Cytotoxicity was expressed by comparison of colonies in treated cultures versus vehicle control cultures (relative cloning efficiency).

2. Treatment protocol without metabolic activation

Exponentially growing V79 cells were plated in 20 mL culture medium in two 75 cm^2 flasks per concentration ($4x10^6$ cells per flask) including all control groups. After attachment (16 to 24 hours later), cells were exposed to vehicle alone or to a range of concentrations of the test substance for 5 hours in 20 mL culture medium with reduced serum content (2%). Thereafter, cell monolayers were washed with PBS, trypsinized and replated in 20 mL culture medium using $1.5x10^6$ cells per 75 cm^2 flask and in 5 mL culture medium using 200 cells per Petri dish (diameter of 60 mm). One flask and 3 Petri dishes were used per culture. These dishes were incubated for 6 to 8 days to allow colony development and to determine the cytotoxicity associated with each test substance directly after treatment (survival to treatment).

Cells in 75 cm^2 flasks were incubated to permit growth and expression of induced mutations. Cells were subcultured (= count 1, normally after 3 days) by reseeding $1.5x10^6$ cells into 20 mL of medium in 75 cm^2 flasks. At the end of the expression period (= count 2, normally a total of 6 days), cultures were reseeded in Petri dishes (diameter of 100 mm) at $3x10^5$ cells per dish (8 dishes per culture) in 20 mL culture medium without hypoxanthine but containing 10 µg/mL 6-TG for selection of mutants. In addition, 200 cells per dish (diameter of 60 mm, 3 dishes per culture) were seeded in 5 mL culture medium to determine the absolute cloning efficiency for each concentration. After incubation for 6 to 8 days, the colonies were fixed, stained with Giemsa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.

Two trials were performed.

C. Treatment protocol with metabolic activation

The activation assay was performed independently. The procedure was identical to the non-activation assay except for the addition of S9 mix. In these experiments 19 mL instead of 20 mL culture medium and additionally 1 mL of S9 mix were added to the flasks for the treatment period, resulting in a concentration of 5% S9 mix in the cultures. The number of 6-TG resistant mutants and viability were determined as in the non-activation assay.

Two trials were performed.

D. Parameters assessed

The parameter "survival to treatment" in % was determined on the basis of the following calculation:

$$\frac{\text{Mean number of colonies (treated cultures)} \times 100}{\text{Mean number of colonies (negative control cultures)}}$$

The "absolute population growth" was calculated using the following formula:

Absolute population growth (for each culture) = cell count 1 x cell count 2

The parameter "relative population growth" shows the cumulative growth of the treated cell populations, relative to the vehicle control.

$$\frac{\text{Absolute population growth treated culture} \times 100}{\text{Absolute population growth of corresponding negative control culture}}$$

The ability of cells to form colonies at the time of mutant selection is measured by the parameter "absolute cloning efficiency". It is expressed in %.

$$\frac{\text{Mean number of colonies per dish} \times 100}{200}$$

The "mutant frequency" is calculated for each group by dividing the total number of mutant colonies by the number of cells seeded (usually 8-10 plates at $3x10^5$ cells per plate), corrected for the absolute cloning efficiency. The mutant frequency is expressed as 6-TG resistant mutants per 10^6 clonable cells.

$$\frac{\text{Total number of mutant colonies x 100}}{\text{Number of evaluated dishes x 3 x } 10^5 \text{ x C.E.}}$$

E. Acceptance criteria

- The average cloning efficiency of the negative and vehicle controls should be at least 50%.
- The average of mutant frequency of the negative controls should not exceed 25 x 10^{-6} cells.
- The mutant frequency of the two cultures of the vehicle and /or the negative control should differ only to an acceptable extent. As a rule of thumb, the difference of mutant frequencies should not be greater than 5 x 10^{-6}.
- The positive control should induce an average mutant frequency of at least three times that of the negative control.
- If not limited by the solubility of the test substance in the vehicle the highest concentration should induce cytotoxicity of about 80 to 90% or should be a concentration where precipitation occurs in the medium. The survival at the lowest concentration should be in the range of the untreated control.
- For the calculation of an acceptable mutant frequency at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency should be 10% or greater.

However, these criteria may be overruled by good scientific judgment.

F. Assessment criteria

- Mutant frequencies was only used for assessment, if at least 5 dishes per culture were available and relative survival to treatment, relative population growth and absolute cloning efficiency were 10% or greater.
- A trial was considered positive if a concentration-related and in parallel cultures reproducible increase in mutant frequencies was observed. To be relevant, the increase in mutant frequencies should be at least two to three times that of the highest negative or vehicle control value observed in the respective trial. If this result was reproduced in a second trial, the test substance was considered to be mutagenic.
- Despite these criteria, a positive result was only considered relevant if no significant change in osmolality compared to the vehicle control was observed. Otherwise, unphysiological culture conditions may be the reason for the positive result.
- A test substance was judged as equivocal if there was no strictly concentration related increase in mutation frequencies but if one or more concentrations induced a reproducible and biologically relevant increase in mutant frequencies in all trials.
- An assay was considered negative if no reproducible and relevant increases of mutant frequencies were observed.

However, these criteria may be overruled by good scientific judgment.

G. Statistical analysis

The statistical analysis relied on the mutant frequencies which were submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights.

The two mutant frequency values obtained per group were, although somewhat related, considered as independent measurements thus increasing the power of the statistical tests applied. Since the protocol of the HPRT assay required at least two independent trials, the overall analysis without respectively with activation was the most important one for classifying substances into mutagens and non-mutagens. However, separate analyses were run for each trial in order to examine the consistency of the results.

All acceptable groups were included in the weighted analysis of variance followed by pair wise comparisons to the negative control on a nominal significance level of $\alpha = 0.05$ using the Dunnett test. The regression analysis part was performed on the basis of the actual concentrations thereby omitting the positive, negative and untreated controls. If there was a significant concentration related increase of the mutant frequency ($\alpha = 0.05$) in the main analysis the highest concentration was dropped and the analysis repeated. This procedure was repeated until $p > 0.05$. In that way eliminated concentrations were flagged correspondingly.

II. Results and discussion

A. General remarks

In the absence and in the presence of S9 mix Chinese hamster V79 cells were exposed to BYI 02960 at concentrations of up to and including 2944 µg/mL. Substance precipitation occurred in the medium at 2944 µg/mL both without and with S9 mix.
The means of the absolute cloning efficiency for the negative controls in the mutation experiments were 75.9% and 89.2% in the experiments without activation. In experiments with metabolic activation, 73.1% and 85.9% cloning efficiencies were observed. These results demonstrate good cloning conditions for the experiments.

B. Mutation assay without metabolic activation

Under non activation conditions two assessable trials were performed.
The mutant frequencies of the untreated controls and of the negative controls were all within the normal range. The positive control EMS induced clear and statistically significant mutagenic effects in all trials.
BYI 02960 was tested up to 2944 µg/mL, a concentration which exceeded the requested limit concentration of 10 mM. For BYI 02960 treated cultures, no cytotoxic effects of 80% to 90% were induced.
Relevant BYI 02960 induced increases in mutant frequencies could not be found. In addition, the overall statistical analysis reveals no statistically significant increases. Therefore, BYI 02960 was evaluated as non-mutagenic in the non-activation trial.

C. Mutation assay with metabolic activation

Two assessable trials6 were performed with S9 mix. The mutant frequencies of the untreated controls and of the negative controls were all within the normal range. The positive control DMBA induced clear mutagenic and statistically significant effects in all trials.
BYI 02960 was tested up to 2944 µg/mL, a concentration which exceeded the requested limit concentration of 10 mM. For BYI 02960 treated cultures, no cytotoxic effects of 80% to 90% were induced.
BYI 02960 induced no relevant increases in mutant frequencies. In addition, the overall statistical analysis reveals no statistically significant increase. With metabolic activation BYI 02960 was therefore evaluated as non-mutagenic.

Table 5.4-07: HPRT assay without metabolic activation - experiment 1

Concentration µg/mL	Survival to treatment (Colony mean)	Population growth (% of NC)	Cloning efficiency (%)	Mutant frequency ($x10^{-6}$)
Untreated control	136	129.7	78.7	1.1
	131	97.9	80.2	9.4
Negative control	121	100	87	5.3
DMSO	128.7	100	91.3	10.0
BYI 02960 46	144	128.4	75.3	0.0
	121.7	106	81.8	5.1
92	152.3	112.2	73.5	14.2
	128	107.8	81.7	4.6
184	135.7	120.8	74.3	6.2
	155	120.6	78.7	10.6
368	114.3	80.6	82.5	1.0
	142.7	135.7	74.3	0.0
736	148	119.8	87.2	5.7
	154	122.9	83.7	4.5
1472	130	114.5	79.2	3.7
	165	144.4	80	3.6
2944	129.3	101.8	73.2	5.1
	63.3	68.8	79	7.4
EMS 900	126.7	46.8	65.5	628.5
	102.3	33.9	71.7	751.7

Table 5.4-08: HPRT assay without metabolic activation - experiment 2

Concentration µg/mL	Survival to treatment (Colony mean)	Population growth (% of NC)	Cloning efficiency (%)	Mutant frequency ($x10^{-6}$)
Untreated control	176.3	62.2	83.2	2.0
	170.7	103.3	78.7	6.4
Negative control	178	100	75.7	2.2
DMSO	206.7	100	78	4.6
BYI 02960 46	163.3	58.7	76.7	6.0
	186.3	91	74.5	5.6
92	160.7	56.3	74.3	1.7
	194	97.4	71.8	4.6
184	167	54.5	81.2	8.7
	174.3	94.9	74.3	9.5
368	147	54.9	74.5	4.5
	147.3	84.9	77.8	1.6
736	171	56.2	82.5	1.5
	149.7	75.8	75.7	4.4
1472	161.3	63.9	72.8	4.6
	160.3	87.8	75.2	3.9
2944	161.3	75	64.3	1.9
	160	60.1	80	6.8
EMS 900	71.3	19.9	75.2	680.7
	90.7	37	79.2	495.8

Table 5.4-09: HPRT assay with metabolic activation - experiment 1

Concentration µg/mL	Survival to treatment (Colony mean)	Population growth (% of NC)	Cloning efficiency (%)	Mutant frequency (x10-6)
Untreated control	182.7	128.3	64.3	9.1
	144.7	130	69.7	10.6
Negative control	173.3	100	68.8	1.2
DMSO	179	100	77.3	1.4
BYI 02960 46	169.7	106.1	70.3	1.8
	172	127	76.7	4.9
92	143	89.4	78.3	1.6
	164.7	124.1	76.7	
184	155.3	103.4	77.5	2.3
	145	137	73	9.7
368	181.7	127.7	81	3.1
	160.7	134.1	73.3	5.7
736	185.3	111.7	71.2	9.4
	166.7	102.2	83.8	6.5
1472	161	90.4	80.7	8.3
	177.3	137.5	80.8	8.2
2944	157.3	98.3	76.7	2.7
DMBA 20	175	57.1	79.7	161.6
	100	22.4	84.5	150.4

Table 5.4-10: HPRT assay with metabolic activation - experiment 2

Concentration µg/mL	Survival to treatment (Colony mean)	Population growth (% of NC)	Cloning efficiency (%)	Mutant frequency (x10-6)
Untreated control	149.3	105.4	68.2	0.6
	161	78.1	83.5	0.5
Negative control	171.7	100	93.8	0.9
DMSO	181.3	100	78	2.1
BYI 02960 46	162.3	114.3	83.3	2.0
	139.3	49.1	82.5	0.5
92	170.7	94.4	82	2.0
	156.3	55.7	67	0.0
184	158.7	114	80.8	2.6
	194.3	46.8	74.7	1.1
368	170.3	99.3	78.5	0.5
	192.3	67.4	79.8	1.6
736	180.3	87.8		
	189.3	38.3	80.7	1.0
1472	187	116.6	82	0.0
	181.3	33.6	93.5	0.9
2944	161.3	82.4	76	2.7
	162.3	48.8	85.7	0.5
DMBA 20	63	22.8	76	121.7
	33.7	12.6	79.5	139.9

III. Conclusions

BYI 02960 was tested up to 2944 µg/mL, a concentration which exceeded the requested limit concentration of 10 mM. Under both activation conditions, no cytotoxic effects were induced. BYI 02960 induced no biologically relevant increases in mutant frequencies.

The positive controls EMS and DMBA had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant frequencies as compared to the corresponding negative controls and thus demonstrated the sensitivity of the test system and the activity of the used S9 mix.

Despite this sensitivity, there were no indications of mutagenic effects of BYI 02960 in the V79/HPRT forward mutation assay either without or with S9 mix.

KIIA 5.4.4 - *In vivo* genotoxicity (somatic cells) - Bone marrow or micronucleus

Report:	**KIIA 5.4.4/01, [redacted] B.; 2009**
Title:	BYI 02960, Micronucleus test on the male mouse
Report No & Document No	AT05350 M-353785-01-2
Guidelines:	OECD 474 (1997); EEC Directive 2000/32/EC Method B12 (2000); EPA Health Effects Test Guideline (OPPTS 870.5395; 1998)
GLP	Yes (certified laboratory)

Executive Summary

BYI 02960 was tested for genotoxicity in an *in vivo* mouse micronucleus assay. In a range finding study with male and female mice (3 per sex per dose), the following doses were used: 10mg/kg, 40mg/kg, 100mg/kg and 1000mg/kg BYI 02960. In both males and females the following symptoms were recorded at all doses: apathy, sternal recumbency, spasm, periodically stretching of body and difficulty in breathing. Symptoms in surviving animals were recorded for up to at least 24 hours after the second application. In addition, all males and all females died in the 100 mg/kg and in the 1000 mg/kg group. No animals died at lower doses.
Based on these findings, a dose level of 40 mg/kg BYI 02960 was chosen as the MTD for males. Based on the results of the dose range finder it was concluded, that there are no substantial differences between sexes in toxicity. Therefore, no females were used in the definitive study.

In the definitive study, groups comprised of 5 male mice were treated at dose levels of 10, 20 and 40 mg/kg of BYI 02960. Five additional animals were treated at 40 mg/kg of BYI 02960 in case of mortality in the initial group or need of replacement of slides due to pathological altered ratio of polychromatic to normochromatic erythrocytes. The BYI 02960 treated groups received two intraperitoneal injections separated by 24 hours. The negative control group was treated with two intraperitoneal injections of corn oil and the positive control group received only one intraperitoneal injection of cyclophosphamide at 20 mg/kg.

Bone marrow from at least one femur from each animal was sampled 24 hours after the last intraperitoneal injection. Slides of bone marrow cells were prepared and scored for the occurrence of micronucleated polychromatic erythrocytes (micronucleated PCEs), of micronucleated normochromatic erythrocytes and PCE/total erythrocytes ratios. 2000 polychromatic erythrocytes were counted per animal.
Males treated twice with BYI 02960 showed symptoms of toxicity after administration at all dose levels. However, all males survived until the end of the test. There was no altered ratio between polychromatic and normochromatic erythrocytes. After two intraperitoneal treatments of males at doses up to and including 40 mg/kg no indications of a clastogenic effect of BYI 02960 were found. Cyclophosphamide, the positive control, had a clear clastogenic effect, as is shown by the biologically relevant increase in polychromatic erythrocytes with micronuclei. The ratio of polychromatic to normochromatic erythrocytes was not altered.

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered BYI 02960 in the micronucleus test on the male mouse, *i.e.* in a somatic test system *in vivo*.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Fine light-brown powder
Lot/Batch:	Batch 2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable for the duration of the study
2. Control materials:	
Negative:	None
Solvent:	Corn oil
Positive:	Cyclophosphamide used in form of Endoxan 100 mg injection vials of dry substance (Baxter Oncology GmbH) dissolved in physiological saline solution
3. Test animals:	
Species:	Mouse
Strain:	Crl: NMRI BR,
Age:	6 to 12 weeks approximately
Weight at dosing:	37 to 43 g (males only)
Source:	[redacted]
Number of animals per dose:	Range-finding test: 3 animals/sex Micronucleus assay: 5 males/group
Animal husbandry:	The animals were properly maintained
4. Test compound concentrations:	
Range-finding test:	2 intraperitoneal injections of 10mg/kg, 40mg/kg, 100mg/kg and 1000mg/kg separated by 24 hours
Micronucleus assay:	0, 10, 20 and 40 mg/kg

B. Test performance

[redacted]) conducted the study during the period March 31 to June 2, 2009.

1. Treatment and sampling times

Sampling took place 24 hours after the last intraperitoneal injection; the positive control was sampled at 24 hours after the only one intraperitoneal injection.

2. Tissues and cells examined

Bone marrow; 2 000 polychromatic erythrocytes (PCEs) examined per animal; the number of normochromatic eryhtrocytes (NCEs, more mature RBCs) per 2 000 PCEs was noted.

3. Details of slide preparation

At 24 hours after the second intraperitoneal injection of BYI 02960 or vehicle control, or 24 hours after the single intraperitoneal injection of positive control, the appropriate groups of animals were sacrificed. Bone marrow smears were prepared from at least one intact femur for each animal. Cell smears were prepared and stained according to conventional cytological procedures.

Coded slides were scored for the presence of micronuclei in 2000 PCEs per animal. The ratio of PCEs to NCEs was also recorded for each animal, as an indication of cytotoxicity to the target tissue. The number of normochromatic erythrocytes showing micronuclei was also established.

4. Evaluation criteria

A test was considered positive if there was a relevant and significant increase in the number of polychromatic erythrocytes showing micronuclei in comparison to the negative control.

A test was considered negative if there was no relevant or significant increase in the rate of micronucleated polychromatic erythrocytes. A test was also considered negative if there was a significant increase in that rate which, according to the laboratory's experience was within the range of historical negative controls.

In addition, a test was considered equivocal if there was an increase of micronucleated polychromatic erythrocytes above the range of attached historical negative controls, provided the increase was not significant and the result of the negative control was not closely related to the data of the respective treatment group. A test was also considered equivocal if its result was implausible. In both cases, normally a second test will be performed.

5. Statistical methods

The BYI 02960 group(s) with the highest mean (provided this superceded the negative control mean) and the positive control were checked by Wilcoxon's non-parametric rank sum test with respect to the number of micronucleated polychromatic erythrocytes and the number of normochromatic erythrocytes.

The rate of normochromatic erythrocytes containing micronuclei was examined if the micronuclear rate for polychromatic erythrocytes was already relevantly increased. In this case, the group with the highest mean was compared with the negative control using the one-sided Chi^2-test.

II. Results and discussion

A. Range-finding test

The following doses were used: 10mg/kg, 40mg/kg, 100mg/kg and 1000mg/kg BYI 02960. In males and females the following symptoms were recorded at all dose levels: apathy, sternal recumbency, spasm, periodically stretching of body and difficulty in breathing. Symptoms in surviving animals were recorded for up to at least 24 hours after the second application. In addition, all mates and all females died in the 100 mg/kg and in the 1000 mg/kg group. No animals died at lower doses.

Based on these findings, 40 mg/kg BYI 02960 was chosen as the MTD for males. Due to the results of the dose range finder it was concluded, that there were no substantial differences between sexes in toxicity. Therefore, no females were used in the definitive study.

B. Micronucleus assay

1. Toxicity

After two intraperitoneal administrations of 10, 20 and 40 mg/kg BYI 02960 treated males showed the following compound-related symptoms: apathy, roughened fur, loss of weight, sternal recumbency, spasm, periodically stretching of body and difficulty in breathing. Symptoms were recorded until sacrifice. These symptoms demonstrate relevant systemic exposure of males to BYI 02960. There was no substance induced mortality. No symptoms were recorded for the control groups. No animals died in these groups.

2. PCE ratio

The ratio of polychromatic to normochromatic erythrocytes in males was not altered by the treatment with BYI 02960, being 2000: 3800 (1s = 1176) in the negative control, 2000:3134 (1s = 1213) in the 10 mg/kg group, 2000:3139 (1s = 1543) in the 20 mg/kg group and 2000:3008 (1s = 878) in the 40 mg/kg group. No relevant variations were thus noted for males.

3. Micronucleated polychromatic erythrocytes

No biologically important or statistically significant variations existed for males between the negative control and the groups treated intraperitoneally with BYI 02960 with respect to the incidence of micronucleated polychromatic erythrocytes. The incidence of these micronucleated cells was 3.4/2000 (1s = 2.7) in the negative control, and 1.4/2000 (1s = 1.5), 3.0/2000 (1s = 1.2) and 3.8/2000 (1s = 2.3) in the BYI 02960 groups.

There would be no biologically significant variation expected between the negative control and BYI 02960 groups in the number of micronucleated normochromatic erythrocytes, since normochromatic erythrocytes originated from polychromatic ones. As expected, relevant variations were not observed.

The positive control, cyclophosphamide, caused a clear increase in the number of polychromatic erythrocytes with micronuclei. The incidence of micronucleated cells was 20.6/2000 (1s = 8,6), which represents biologically relevant increases in comparison to the negative control.

No further effect of cyclophosphamide was found concerning the ratio of polychromatic to normochromatic erythrocytes, since this ratio did not vary to a biologically relevant degree [2000: 3308 (1s = 1364), as against 2000: 3800 in the negative control]. This clearly demonstrates that an alteration of the ratio of polychromatic to normochromatic erythrocytes is not necessary for the induction of micronuclei.

Table 5.4-10: Summary of micronucleus test results

Test group	Dose mg/kg bw	Sampling time (h)	Number of NCE per 2000 PCE	MNNCE per 2000 NCE	MNPCE per 2000 PCE
Vehicle	0	24	3800	1.5	3.4
BYI 02960	2 x 10	24	3134	1.0	1.4
	2 x 20	24	3139	1.9	3.0
	2 x 40	24	3008	1.0	1.8
Cyclophosphamide	20	24	3308	1.9	20.6**

** : p≤0.01
NCE : normochromatic erythrocytes PCE : polychromatic erythrocytes
MNNCE : micronucleated NCE MNPCE : micronucleated PCE

III. Conclusions

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered BYI 02960 in the micronucleus test on the male mouse, *i.e.* in a somatic test system *in vivo*.

Report:	**KIIA 5.4.4/02, [redacted] J., 2011**
Title:	Micronucleus assay in bone marrow cells of the mouse with BYI 02960-a.i.
Report No & Document No	1425801 M-420536-01-2
Guidelines:	OECD 474 (1997); EEC Directive 440/2008/B.12 (2008); EPA Health Effects Test Guideline (OPPTS 870.5395, 1998)
GLP	Yes (certified laboratory) except that no analytical analyses were performed during the study

Executive Summary

This study was performed to test the specification required for production of the technical BYI 02960 and to fulfill Chinese requirements asking for testing both sexes in the *in vivo* micronucleus assay. Therefore only female mice were used in this study as males had been tested in the above study (M-353785-01-2). The following doses were used in a pre-experiment: 10mg/kg, 50mg/kg, 75mg/kg and 100mg/kg BYI 02960. Two females were used per dose group. All females died in the 100 mg/kg group and one female died and the second one was euthanized in the 75 mg/kg group. No animals died at lower doses. Reduced spontaneous activity and ruffled fur were observed in both females in the 50 mg/kg group after the first and the second application; abdominal position was seen shortly after the second application. At 10 mg/kg reduced spontaneous activity, eyelid closure and ruffled fur were observed only after the second application. Based on these findings, 50 mg/kg BYI 02960 were chosen as MTD for females. Based on these findings, 12.5, 25 and 50 mg/kg were chosen as dose levels for the main experiment.

In the main experiment, each group comprised 7 female mice. The negative control group was treated with two intraperitoneal injections of 10% DMSO/90% Corn oil and the positive control group received only one intraperitoneal injection of cyclophosphamide at 40 mg/kg. BYI 02960 treated groups also received two intraperitoneal injections separated by 24 hours.

Sampling of the bone marrow was done 24 hours after the last intraperitoneal injection. Slides of bone marrow cells were prepared and scored for the occurrence of micronucleated polychromatic erythrocytes (micronucleated PCEs), of micronucleated normochromatic erythrocytes and PCE/total erythrocytes ratios. 2000 polychromatic erythrocytes were counted per animal.
Females treated twice with BYI 02960 in doses up to 50 mg/kg showed symptoms of toxicity after administration, starting at 25 mg/kg. These symptoms demonstrate relevant systemic exposure of females to BYI 02960. However, all females survived until the end of the test. After treatment with BYI 02960 the number of PCEs was not substantially decreased as compared to the mean value of PCEs of the vehicle control group thus indicating that BYI 02960 did not exert any cytotoxic effects in the bone marrow. After two intraperitoneal treatments of females with doses up to and including 50 mg/kg no indications of a clastogenic effect of BYI 02960 were found.
Cyclophosphamide, the positive control, had a clear clastogenic effect, as is shown by the biologically relevant increase in polychromatic erythrocytes with micronuclei.

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered BYI 02960 in the micronucleus test on the female mouse, *i.e.* in a somatic test system *in vivo*.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	PFV107N005
Purity:	97.2%
CAS:	951659-40-8
Stability of test compound:	No analysis performed during the study, formulations prepared on the day of the experiment
2. Control materials:	
Negative:	None
Solvent:	10% DMSO/90% corn oil
Positive:	Cyclophosphamide (Fisher Scientific, Germany) dissolved in sterile water
3. Test animals:	
Species:	Mouse
Strain:	NMRI
Age:	8 to 9 weeks approximately
Weight at dosing:	Mean weight 1st application 28.6g Mean weight 2nd application 28.4g
Source:	[redacted]
Number of animals per dose:	Range-finding test: 2 females/group Micronucleus assay: 7 females/group
Animal husbandry:	The animals were properly maintained

4. Test compound concentrations:

Range-finding test:	2 intraperitoneal injections of 10mg/kg, 50mg/kg, 75mg/kg and 100mg/kg separated by 24 hours
Micronucleus assay:	0, 12.5, 25 and 50 mg/kg

B. Test performance

The experimental phase of the study was performed between July 12 to August 17, 2011 [redacted] Germany.

1. Treatment and sampling times

The animals received BYI 02960 or the vehicle twice intraperitoneally and the positive control substance once intraperitoneally. The administered volume was 10 mL/kg bw. Seven females were treated per dose group. The animals of all groups (excepted the positive control) were examined for acute toxic symptoms at intervals of approximately 1 h, 2-4 h, 6 h and 24 h after each administration of BYI 02960 and the vehicle. Sampling of the bone marrow was done 24 hours after the last treatment.

2. Tissues and cells examined

Bone marrow; 2 000 polychromatic erythrocytes (PCEs) examined per animal; the number of normochromatic eryhtrocytes (NCEs, more mature RBCs) per 2 000 PCEs was noted.

3. Details of slide preparation

At 24 hours after the second intraperitoneal injection of BYI 02960 or vehicle control, or 24 hours after the only one intraperitoneal injection of positive control, the appropriate groups of animals were sacrificed using CO_2 followed by bleeding. Bone marrow smears were prepared for each animal. Cell smears were prepared and stained according to conventional cytological procedures.

Coded slides were scored for the presence of micronuclei in 2000 PCEs per animal. The ratio of PCEs to NCEs was also recorded for each animal, as an indication of cytotoxicity to the target tissue.

4. Evaluation Criteria

A test item is classified as mutagenic if it induces either a dose-related increase or a clear increase in the number of micronucleated polychromatic erythrocytes in a single dose group. Statistical methods (nonparametric Mann-Whitney test were used as an aid in evaluating the results). However, the primary point of consideration is the biological relevance of the results.

A test item that fails to produce a biological relevant increase in the number of micronucleated polychromatic erythrocytes is considered non-mutagenic in this system.

II. Results and discussion

A. Range-finding test

The following doses were used in a pre-experiment: 10mg/kg, 50mg/kg, 75mg/kg and 100mg/kg BYI 02960. Two females were used per dose group. All females died in the 100 mg/kg group and one

female died and the second one was euthanized in the 75 mg/kg group. No animals died at lower doses. Reduced spontaneous activity and ruffled fur were observed in both females in the 50 mg/kg group after the first and the second application; abdominal position was seen shortly after the second application. At 10 mg/kg, reduced spontaneous activity, eyelid closure and ruffled fur were observed only after the second application. Based on these findings, 50 mg/kg BYI 02960 were chosen as MTD for females. 12.5, 25 and 50 mg/kg were chosen as dose levels for the main experiment.

B. Micronucleus assay

1. Toxicity

The following clinical signs were observed in the females treated at 50 mg/kg after the first or the second administration: reduced motor activity, ruffled fur, abdominal position and eyelid closure. In the animals treated at 25 mg/kg, reduced motor activity, eyelid closure and ruffled fur were observed between 2 and 4 hours after the first administration only. The animals treated at 12.5 mg/kg did not present any clinical signs.

2. Number of PCE per 2000 erythrocytes

After treatment with BYI 02960, the number of PCEs was not substantially decreased as compared to the mean value of PCEs of the vehicle control group thus indicating that BYI 02960 did not exert any cytotoxic effects in the bone marrow: 1229 in the controls, 1250 at 12.5 mg/kg, 1234 at 25 mg/kg and 1177 at 50 mg/kg.

3. Micronucleated polychromatic erythrocytes

In comparison to the corresponding vehicle control values there was no biologically relevant enhancement in the frequency of the detected micronuclei at any preparation interval and dose level after administration of BYI 02960. The mean values of micronuclei observed after treatment with BYI 02960 were near or below to the value of the vehicle control group. Additionally no clear dose dependence could be observed and, thus this isolated effect is considered to be not biologically relevant.

Cyclophosphamide administered intraperitoneally (40 mg/kg; 10 mL/ kg, once) was used as positive control which showed a substantial increase of induced micronucleus frequency.

Table 5.4-11: Summary of micronucleus test results

Test group	Dose mg/kg bw	Sampling time (h)	PCEs with micronuclei	Range	PCE per 2000 erythrocytes
Vehicle	0	24	0.121	0 - 5	1229
BYI 02960	12.5	24	0.114	1 - 5	1250
	25	24	0.143	1 - 5	1234
	50	24	0.093	0 - 3	1177
Cyclophosphamide	40	24	2.264	32 - 63	1125

III. Conclusions

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered BYI 02960 in the micronucleus test on the female mouse.

KIIA 5.4.5 - *In vivo* genotoxicity (somatic cells) - DNA repair or mouse spot tests

Based on the results of the *in vitro* and *in vivo* studies as reported under points 5.4.1 to 5.4.4, no further studies in germ cells were triggered.

KIIA 5.4.6 - *In vivo* studies in germ cells

Based on the results of the *in vitro* and *in vivo* studies as reported under points 5.4.1 to 5.4.4, no further studies in germ cells were triggered.

KIIA 5.5 - Long-term toxicity and carcinogenicity

The oncogenic potential of BYI 02960 was assessed in both the rat and the mouse. The studies were conducted between 2009 and 2012. All were in accordance with OECD, EU, USEPA and Japanese MAFF testing guidelines and were fully compliant with GLP. A summary of these results is presented in Table 5.5-01.

In the rat combined chronic toxicity and carcinogenicity study, where the animals were administered BYI 02960 through the diet at up to 2000 ppm, lower body weight and body weight gain were observed in females at 2000 ppm throughout the study and slightly lower cumulative body weight gain was observed in males during the first year. Higher mean leukocyte counts associated with higher mean absolute lymphocyte and neutrophil counts were observed in males from the end of the first year. Slightly higher cholesterol concentrations were seen in the females throughout the study. No relevant treatment-related neoplastic changes were observed at any dose level tested. The target organs were the liver and the thyroid in either sex and the lung in females. The effects seen in the liver in the 2000 ppm treated male and female groups were higher mean liver to body weight ratios associated with centrilobular hypertrophy, centrilobular hepatocellular macrovacuolation, lower incidences of periportal hepatocellular vacuolation and eosinophilic, mixed and tigroid foci of altered hepatocytes. In addition, higher incidences of brown pigments in Kupffer cells, interstitial mononuclear cell infiltrate and periportal hepatocellular macrovacuolation were observed in females. Changes were also observed in the thyroid gland including higher incidences of follicular cell hypertrophy and of follicular cell pigment in both sexes at the final sacrifice and increased incidences of colloid alteration in males and females at the interim sacrifice and in males only at final sacrifice. In the lung, higher incidences of foamy macrophages and chronic interstitial and perivascular inflammation were observed in females at final sacrifice. At 400 ppm, the findings were limited to centrilobular hypertrophy (minimal) in the liver and of colloid alteration in the thyroid gland observed in males. However these changes were considered not to be adverse since they were minimal and/or not associated with other relevant changes. The No Observed Adverse Effect Level (NOAEL) over a 12-month period of dietary administration with BYI 02960 to the Wistar rat was 400 ppm in both sexes (equivalent to 18.5 mg/kg body weight/day in males and 25.3 mg/kg body weight/day in females).
The No Observed Adverse Effect Level (NOAEL) over a 24-month period of dietary administration with BYI 02960 to the Wistar rat was 400 ppm in both sexes (equivalent to 15.8mg/kg/day weight/day in males and 22.5 mg/kg body weight/day in females).

In the mouse carcinogenicity study, where animals were administered BYI 02960 through the diet up to 1500 ppm, mean body weight was progressively decreased in both sexes throughout the study compared to control means. No relevant treatment-related neoplastic changes were observed at any dose level tested. The target organs were the liver and the kidney. The changes observed in the liver were higher liver weights and a higher incidence and severity of diffuse hepatocellular vacuolation (mainly centrilobular) noted in males, whilst a decreased incidence of diffuse hepatocellular macrovacuolation (mainly periportal) was noted in females. In the kidney, lower weight, decreased incidence and severity of bilateral basophilic tubules, focal cortical mineralization and corticoepithelial vacuolation were noted in males. These changes in the kidney or the liver were considered to be treatment-related but not adverse. At 300 ppm, the only histopathological changes were noted in males and consisted of a higher incidence and severity of diffuse hepatocellular vacuolation (mainly centrilobular) in the liver together

with a decreased severity of corticoepithelial vacuolation in the kidney. Both changes were considered to be treatment-related but not adverse.
A dose level of 300 ppm (equivalent to 43 mg/kg/day in males and 53 mg/kg/day in females) was considered to be a No Observed Adverse Effect Level (NOAEL) in both sexes over an 18-month period of dietary administration.

Table 5.5-01: Summary of long-term toxicity/carcinogenicity with BYI 02960

Type of study Doses	NOAEL mg/kg/day	LOAEL mg/kg/day	Effects
Rat - 104-week Chronic Toxicity/ Oncogenicity M-428257-01-1 80, 400 & 2000 ppm	15.8/22.5 (M/F)	80.8/120 (M/F)	Target organs: liver & thyroid either sex; lung in females No tumours
Mouse - 78 week- Chronic/ Oncogenicity M-425975-01-1 70, 300 & 1500 ppm	43/53 (M/F)	224/263 (M/F)	Target organs: liver either sex; kidney in males No tumours

KIIA 5.5.1 - Long-term (2 years) oral toxicity in the rat

The long term (2 years) oral toxicity of BYI 02960 in rats was investigated in a combined chronic/carcinogenicity study reported under point IIA 5.5.2.

KIIA 5.5.2 - Carcinogenicity study in the rat

Report:	**KIIA 5.5.2/01 [redacted] J.-C.; 2012**
Title:	BYI 02960, Chronic toxicity and Carcinogenicity study in the Wistar rat by dietary administration
Report No & Document No	SA 08337 M-428257-01-1
Guidelines:	OECD 453 (1981); EEC Directive 88/302/EEC – Annex V - Method B.33. (1987); EPA Health Effects Test Guideline (OPPTS 870.4300, 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
GLP	Yes (certified laboratory)

Executive Summary

Groups of 70 male and 70 female rats were fed diet containing 0, 80, 400 and 2000 ppm BYI 02960 (batch number 2009-000239, a beige powder, 96.2% w/w). After 52 weeks, 10 males and 10 females from each group allocated to the chronic (12 month) phase were necropsied at the scheduled interim sacrifice. The remaining 60 animals/sex/group, allocated to the carcinogenicity (24-month) phase of the study, continued treatment until final sacrifice of the study after at least 104 weeks of treatment, when surviving animals were necropsied. Mortality and clinical signs were checked daily. Detailed physical examinations including palpation for masses were performed at least weekly throughout the study. Body weight was recorded weekly for the first 13 weeks, then approximately every 4 weeks thereafter. Food consumption was recorded twice weekly for the first 6 weeks of the study, then approximately weekly up to Week 13, then every 4 weeks thereafter. Ophthalmology examinations were performed on all animals during acclimatization and after approximately 24 months. In addition, ophthalmology examinations were performed on all animals from the control and high dose groups after approximately 12 months. Hematology and clinical chemistry determinations and urinalysis were performed during months 4, 6, 12, 18 and 24 on selected animals. At the scheduled chronic and carcinogenicity phase sacrifices, selected organs were weighed and designated tissues sampled and examined microscopically.

<u>At 2000 ppm</u>
The overall incidence and percentage of mortality was comparable with controls in males throughout the study, whereas there was a lower mortality rate in females compared to controls.
During the first year of treatment, only a few clinical signs were observed including slightly higher incidences than controls of soiled fur, hyper-reactivity to external stimuli and resistance to handling in males and higher incidence of hair loss in females. During the second year of treatment, hair loss and soiled fur were observed with slightly higher incidences in males compared to controls.
In females, mean body weight and cumulative body weight gains were lower compared to controls throughout the study (- 5% to - 17% for body weight and - 18% to - 62% for cumulative body weight gains). Overall, the mean cumulative body weight gain was decreased by 23% when compared to controls, and at the end of the study the mean body weight was 13% lower. In males, the mean cumulative body weight gain was significantly lower than controls during the first week of treatment (- 27%) and over the first three months of the study (- 13%) and comparable to controls thereafter. The

mean body weight was lower compared to controls from week 1 to week 54 (statistically significant in most occasions) and comparable to controls thereafter.
Overall mean food consumption was slightly lower during the first few days of treatment in both sexes. Thereafter it was considered to be comparable to controls in males whereas it was marginally lower than in controls in females on many occasions throughout the study.
Ophthalmological examinations revealed lens opacity in 10/70 females compared to 3/69 in controls after one year. After two years, slightly higher incidences of lens opacity, of iris mydriasis and of a pale color of retinal fundus were observed in females compared to controls. However, lens opacity is a very common finding for rats of this strain and age and was observed with incidence that was only slightly marginally outside the range of our historical control data.
Higher mean leucocyte counts were noted in males, relative to the controls, at Month 12 (+ 22%), 18 (+ 31%) and 24 (+ 63%) associated with higher mean absolute lymphocyte count at Month 12 (+ 19%) and 18 (+ 32%) and with higher mean absolute neutrophil count at Month 12 (+ 36%) and 24 (+ 143%).
Statistically significant differences were noted in some erythrocyte parameters in females, throughout the first year of treatment. However, these changes in females were considered not to be toxicologically relevant in view of their low magnitude and their transient occurrence.

At clinical chemistry examination, relevant change consisted in slightly higher cholesterol concentrations observed in females throughout the study (+ 16% to + 35%, statistically significant during the first year only). Slightly lower glucose concentrations were observed at Month 4 in both sexes and at Month 6 in males only.

At the end of the chronic phase, mean terminal body weight was statistically significantly lower in females compared to controls (- 20%). In both sexes, mean liver to body weight ratios were higher than controls (+ 15% and + 14% in males and females). This was associated with macroscopic changes (enlarged and pale liver) and with histological changes including centrilobular hypertrophy, centrilobular hepatocellular macrovacuolation and lower incidences of periportal hepatocellular vacuolation in both sexes. In addition, higher incidences of eosinophilic and tigroid foci of altered hepatocytes were observed in males. In the thyroid gland, increased incidences and severity of colloid alteration was observed in both sexes.

At the end of the carcinogenicity phase, mean terminal body weight was lower in both sexes (- 7% and - 14% in males and females, respectively). In both sexes, mean liver to body weight ratios were higher than controls (+ 12% and + 10% in males and females, respectively). This was associated with histological changes including eosinophilic, mixed and tigroid foci of altered hepatocytes, centrilobular hypertrophy and hepatocellular macrovacuolation in both sexes. In addition, higher incidences of brown pigments in Kupffer cells, interstitial mononuclear cell infiltrate and periportal hepatocellular macrovacuolation were observed in females.

In the lung, higher incidences of foamy macrophages and chronic interstitial and perivascular inflammation were observed in females.
Changes were also observed in the thyroid gland including higher incidences of follicular cell hypertrophy and of follicular cell pigment in both sexes and increased incidences of colloid alteration in males.

At 400 ppm
With the exception of hair loss which was observed in 13/70 females (compared to 8/70 in controls) during the first year of treatment, no treatment-related clinical signs were observed in either sex. Mean body weight was not affected throughout the study. Mean body weight gain was just statistically significantly lower than controls during the first week of treatment in both sexes (- 6% and - 12% in males and females, respectively) and not affected thereafter. There were no toxicologically relevant changes at the hematology and clinical chemistry evaluation or urinalysis.

At the end of the chronic phase, mean terminal body weight and mean organ weight parameters were unaffected by the treatment in either sex. There was no treatment-related effect at the macroscopic examination in either sex. At the microscopic examination, higher incidences of centrilobular hypertrophy (minimal) in the liver and of colloid alteration in the thyroid gland were observed in males compared to controls. However these changes were considered not to be adverse since they were minimal and/or not associated with other relevant changes.

At the end of the carcinogenicity phase, the only changes were seen at the microscopic examination where higher incidences of centrilobular hypertrophy in the liver and of colloid alteration in the thyroid gland were observed in males compared to controls. However, these changes were considered not to be adverse since they were minimal and/or not associated with other relevant changes.

At 80 ppm
No toxicologically relevant changes were noted throughout the study in either sex for any of the parameters evaluated.

In conclusion, no relevant treatment-related neoplastic changes were observed at any dose level tested.

The No Observed Adverse Effect Level (NOAEL) over a 12-month period of dietary administration with BYI 02960 to the Wistar rat was 400 ppm in both sexes (equivalent to 18.5 mg/kg body weight/day in males and 25.3 mg/kg body weight/day in females).

The No Observed Adverse Effect Level (NOAEL) over a 24-month period of dietary administration with BYI 02960 to the Wistar rat was 400 ppm in both sexes (equivalent to 15.8 mg/kg/day weight/day in males and 22.5 mg/kg body weight/day in females).

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable in rodent diet at 70 and 2500 ppm for up to 110 days when kept at room temperature

2. Vehicle and /or positive control: None

3. Test animals:

Species:	Rat
Strain:	Wistar Rj:WI (IOPS HAN)
Age:	6 weeks approximately
Weight at dosing:	236.5 g - 236.8 g mean group weight males 166.6 g -167.6 g mean group weight females
Source:	[redacted] France.
Acclimation period:	16 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum* except during designated time period
Water:	Filtered and softened tap water, *ad libitum*
Housing:	By sex in groups of 5 unless reduced by mortality or isolation. The cages were suspended stainless steel and wire mesh.
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 55 ± 15% Air changes: Approximately 10 to 15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates:

March 11 2009 to April 19, 2011 at [redacted], France.

2. Animal assignment and treatment

On the day of randomization, animals were allocated to dose groups using a computerized randomization procedure that ensured a similar body weight distribution for each sex. The acceptable body weight range for each sex was ± 20% of the mean body weight on the day of randomization. Any animal deemed unsuitable for selection based on weight, clinical findings or health status was not used for the study.

Animals were assigned to the test groups noted in the following table. Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 5.5.2-01: Study design

Test group	Concentration in diet (ppm)	Dose per animal (week period 1 to 105)		Animals assigned/sex	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Main study 104 weeks	Interim sacrifice 52 weeks
1	0	0	0	50	10
2	80	3.16	4.48	50	10
3	400	15.8	22.5	50	10
4	2000	80.8	120	50	10

During the acclimatization phase, each animal was identified by a micro identification implant from Reseaumatique (Bernay, France).

Dose selection

The dose levels were selected based on the results from a previous 90-day dietary study in the rat, where dietary administration of up to 2500 ppm in males and females resulted in lower body weight throughout the study, lower food consumption and changes in hematology (higher platelet count in females) and clinical chemistry (lower bilirubin and glucose concentrations and higher cholesterol and triglyceride concentrations). In addition, effects were observed in the liver and thyroid gland at pathological examinations. The NOAEL in the rat 90-day study was 500 ppm, with a LOAEL of 2500 ppm.

3. Diet preparation and analysis

The test substance in acetone solution was incorporated into the diet to provide the required dietary concentrations of 80, 400 or 2000 ppm. For control groups, a control formulation was prepared by adding an equivalent volume of acetone into the diet. The test substance formulations were prepared to cover the dietary requirements over at least 4 weekly periods apart from the last formulation which covered the dietary needs until the end of the study. When not in use, the diet formulations were stored at room temperature. The stability of the test substance at 70 and 2500 ppm in the diet was verified for up to at least 110 days, when kept at room temperature which covered the period of storage and usage on this study.

Sixteen formulations were prepared during the study at each concentration. A formulation sequence consisted of three to five loads weighing approximately 54 to 64 kg.

The homogeneity of the test substance in diet was verified from the first loads at 80, 400 and 2000 ppm on the first formulation (F1) and on the first loads at 80 and 2000 ppm of formulations F14 to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. The homogeneity analyses were within 85 to 101% of the nominal concentration.

The concentration was checked for all loads at all dose levels for formulations F1, F4, F7, F10, F14 and F16. The concentration analyses were within 89 to 100% of the nominal concentration.

4. Statistics

Means and standard deviations were calculated for each group and per time period. All statistical analyses were carried out separately for males and females. The Bartlett test was performed to compare the homogeneity of group variances.

If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA), which was followed by Dunnett test (2-sided) if ANOVA indicated significance.

If the Bartlett test was significant ($p \leq 0.05$) (for body weight change parameters, terminal body weight, absolute and relative organ weight parameters, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, % neutrophils, % lymphocytes, % reticulocytes, clinical chemistry parameters, urine volume and refractive index), group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance.

If the Bartlett test was significant ($p \leq 0.05$) (for body weight and average food consumption day parameters), data were transformed using the log transformation. If the Bartlett test on log transformed data was not significant ($p > 0.05$), means were compared using the ANOVA on log transformed data, which was followed by the Dunnett test (2-sided) on log transformed data if ANOVA indicated significance. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided), if the Kruskal-Wallis test indicated significance. Avergae food consumption parameters were analyzed using SAS programs. Levene Test was performed to compare homogeneity of variances.

If the Bartlett test was significant ($p \leq 0.05$) (for haematology parameters such as red blood cell count, platelet count, white blood cell count, neutrophil count, lymphocyte count), data were transformed using the square root transformation. If the Bartlett test on square root transformed data was not significant ($p > 0.05$), means were compared using the ANOVA on square root transformed data, which was followed by the Dunnett test (2-sided) on square root transformed data, if ANOVA indicated significance.

If the Bartlett test was significant ($p \leq 0.05$) even after square root transformation, group means were compared using the non-parametric Kruskal-Wallis which was followed by the Dunn test (2-sided), if Kruskal-Wallis test indicated significance.

For quantitative urinalysis parameter (pH), group means were compared using the non-parametric Kruskal-Wallis which was followed by the Dunn test (2-sided), if Kruskal-Wallis test indicated significance.

If one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5%, 1% and 0.1% levels of significance except for analyses conducted with S.A.S. programs where only 5% and 1% levels of significance were used. Statistical analyses were carried out using Pristima version 6.1.0 build 19 except for average food consumption/day parameter which was analyzed using S.A.S. programs.

For survival analysis

Adjusted survival rates were estimated using Kaplan-Meier estimation procedures. Kaplan-Meier estimates were calculated separately for each sex and treatment group. Mortalities which were the result

of animals dead at anesthesia, killed following accidents (accidental trauma) or at scheduled sacrifices were considered to be censored observations.
Statistical significance of differences in survival rates between treated and control groups and dose-related trends in survival was assessed using Cox's and Tarone's tests. Probability values presented were two-sided for pair wise comparisons and trend test. Group mortality rates were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using SAS programs version 9.2 and LTA.EXE program. Survival analyses were performed on the carcinogenicity phase.

Neoplastic and non-neoplastic findings analyses
When the number of lesion-bearing animals was less or equal to 2 in one group and equal to 0 in the other groups, no statistical test was performed. When the incidences of the 80 and 400 ppm treated groups were equal to 0, only the 2000 ppm treated group was compared to the control group and no trend test was performed.

Not adjusted analyses
Selected lesions were analyzed by Cochran-Armitage method for trend (1-sided) and the Fisher's exact test (1-sided) for control versus treatment comparisons. Cochran-Armitage trend significance is reported when there is existence of any monotone response in the incidence data (that is there is a relationship between the response and the dose represented by a regression line that is continually increasing (or decreasing), but perhaps not in a straight line).

Survival adjusted analyses
Further survival adjusted analyses, considering any possible intercurrent mortality differences due to the competing toxicity among the treated groups, were performed on lesions.
For non-palpable tumors, each tumor was categorized as fatal if the tumor was a factor contributing towards the death of the animal, incidental otherwise.
Incidental tumors and non-neoplastic lesions data were analyzed by logistic regression of tumors prevalence. Logistic regression analysis is based on the assumption that the diagnosed lesions were not directly responsible for the animal's death. Treated and control group lesion rates and dose-related trends were compared using the corrected score test.
Fatal tumors were analyzed by the life-table test. The life-table test is based on the assumption that all lesions were fatal. Statistical significance of differences in incidences between treated and control groups and dose-related trends were investigated using Cox's and Tarone's tests.

Trend tests were conducted firstly including all groups. When both the trend test including all the dose levels and only the high dose group were significant, a second trend test excluding the high dose group was also done.

The reported results reflect 1-sided testing.
Statistical analyses were conducted using SAS programs (Version 9.2), LTA.EXE and LOPRAN.EXE programs.
All tissues missing, autolytic and non-readable, inadequate did not contribute to the analyses.

Group incidences were compared at the 5% and 1% levels of significance.
All finding analyses were performed on the carcinogenicity phase.

C. Methods

1. Clinical signs and mortality
Animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily for all animals. The nature, onset, severity, duration and recovery of clinical signs were recorded. Cages and cage trays were inspected daily for evidence of ill health such as blood or loose feces. Detailed physical examinations including palpation for masses were performed weekly from study Day 1. The onset, location and dimension of the masses were recorded.

2. Neurological examinations
As the neurotoxicological potential of BYI 02960 was examined in other studies, these determinations were not conducted in this study.

3. Body weight
Each animal was weighed at least weekly during the acclimatization period then weekly for the first 13 weeks of study, approximately every 4 weeks thereafter and prior to necropsy. Body weights recorded prior to necropsy are referred to terminal body weights.

4. Food consumption and test item intake
The weight of food supplied and of that remaining at the end of the food consumption period was recorded for each animal. Food consumption was recorded twice weekly during the first 6 weeks of treatment, then weekly up to Week 13, and once approximately every 4 weeks thereafter.
The weekly mean achieved dosage intake in mg/kg body weight/day was calculated for Weeks 1 to 13, then 1 week per month thereafter.
The monthly and overall mean achieved dosage intake for the 24 months of treatment were derived from the weekly data.

5. Ophthalmological examination
During the acclimatization phase, all animals were examined by indirect ophthalmoscopy. During the treatment period, fundoscopic (indirect ophthalmoscopy) and biomicroscopic (slit lamp) examinations were performed on all surviving animals from control and high dose groups after approximately 12 months and from all surviving animals after 24 months of treatment with BYI 02960. Each eye was examined by direct ophthalmoscopy in the first instance, and then after instillation of an atropinic agent (Mydriaticum, Merck Sharp and Dohme), each eye was re-examined by means of a slit lamp and an indirect ophthalmoscope.

6. Hematology and clinical chemistry
Blood was sampled from isoflurane anesthetized animals by puncture of the retro orbital venous plexus after overnight diet fasting. Blood was collected in tubes containing EDTA for hematology, clot activator (for serum) for clinical chemistry and sodium citrate for coagulation.
At terminal sacrifice, blood smears were prepared for all surviving animals.
When possible, a blood smear was prepared for the moribund animals, just before sacrifice.

Blood analyses were performed on all the surviving animals of the 12-month interim sacrifice group on Weeks 14, 26 or 27, 52 or 53.
Blood analyses were performed on the first ten suitable surviving rats of the terminal sacrifice groups on Weeks 14, 26 or 27, 52 or 53, 78 and 104 or 105.
An additional blood sampling was performed in tubes containing sodium citrate for coagulation (0.9 mL) on study Day 371 (April 01, 2010) to replace clotted samples.

Hematology

Red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count were assayed using an Advia 120 (Siemens, Eragny, France). A blood smear was prepared and stained using May-Grünwald-Giemsa method. It was examined when the results of Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL Elite Pro (Instrumentation Laboratory, Paris, France).

Clinical chemistry

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus concentrations, and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed on plasma samples, total protein and albumin concentrations were assayed on serum samples using an Advia 1650 (Siemens, Eragny, France). Globulin concentrations and albumin/globulin ratio values were calculated.

7. Urinalysis

Urinalysis was performed on all surviving animals of the 12-month interim sacrifice group on Weeks 15, 25 or 26 and 50 or 51.

Urinalysis was performed on the first ten suitable surviving rats of the terminal sacrifice groups on Weeks 15, 25 or 26, 50 or 51, 77 or 78 and 103 or 104.
Diet and water were withdrawn during the overnight (approximately 16 hours) collection period.
Urine samples were weighed to determine urinary volume. pH was assayed using a Clinitek 500 and Multistix dipsticks (Siemens, Eragny, France). Urinary refractive index was measured using a RFM 320 refractometer (Bioblock Scientific, Illkirch France).
Glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen were assayed using a Clinitek 500 and Multistix dipsticks (Siemens, Eragny, France). Microscopic examination of the urinary sediment was performed after centrifugation of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was recorded.

8. Bioanalytical evaluations

During Weeks 52 and 105 of the study, a blood sampling (approximately 500 µL) was collected from the retro-orbital venous plexus of five suitable animals from groups 2, 3 and 4 for each sex. Similarly, a blood sampling was also collected from 2 suitable male animals of Group 1 (controls). Animals were not overnight dietary fasted before blood sampling. The time point for blood collection (approximately 9:00 a.m.) was determined based on the results of a previous blood kinetic study conducted in Wistar rats.

Plasmas were prepared from blood collected into heparinized vials by centrifugation and stored in the dark at approximately -20 °C until shipment on dry ice (at approximately -70 °C) for determination of test substance level and potentially its main metabolites at "Bioanalytical Investigations, Bayer CropScience AG, Development, Human Safety - Residue Analysis, [redacted] Germany" under the supervision on the Principal Investigator [redacted]. Shipping procedures followed the Standard Operating Procedures (SOPs) of the Testing Facility.

The samples were analyzed by High Performance Liquid Chromatography/Tandem Mass Spectometry for concentrations of the active substance.

9. Sacrifice and pathology

On study Days 369 to 371 for the 12-month interim kill and on study Days 739 to 753 for the carcinogenicity phase, all surviving animals dedicated to the interim sacrifice/chronic group and carcinogenicity phase group, respectively, were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day at the interim or terminal sacrifices. Animals were diet fasted overnight prior to sacrifice.

All animals, including animals either found dead or killed for humane reasons, were necropsied. The necropsy included the examination of external surfaces, all orifices, all major organs, tissues and body cavities. All significant macroscopic abnormalities (including masses and their regional lymph nodes when possible) were recorded, sampled and examined microscopically.
The following organs were weighed: adrenal gland, brain, epididymides, heart, kidney, liver, ovary, pituitary gland, prostate, spleen, testis, thymus and uterus.
The following organs or tissues were sampled: adrenal gland, aorta, articular surface femorotibial joints, bone (sternum/femur), bone, brain, epididymis, oesophagus, lachrymal gland, eye and optic nerve, harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes, mammary gland, nasal cavities, ovary, pancreas, parathyroids, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary gland, testis, thymus, thyroid gland, tongue, trachea, urinary bladder, uterus (with cervix), ureters, vagina.
Two femoral bone marrow smears were prepared from sacrificed animals (except on weekends and public holidays), one of which was stained with May-Grünwald Giemsa, but not examined as no relevant changes were observed in hematology or bone marrow histology. The second smear was stored unstained.
Samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye and optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative.
All the above samples (except exorbital lachrymal gland, larynx/pharynx and nasal cavities) were embedded in paraffin wax.
For the 12-month interim sacrifice, histological sections, stained with hematoxylin and eosin, were prepared from all organs and tissue samples listed below.

For the carcinogenicity phase, histological sections, stained with hematoxylin and eosin, were prepared from all organs and tissue samples.

Histotechnology from the final sacrifice group animals was performed at:
[redacted]

Histopathology examinations from interim sacrificed animals (52 weeks of treatment) were performed as follows:

- all organs and tissue samples from animals sacrificed or dying during the treatment period,
- all organs and tissue samples from animals of control and high dose groups,
- liver, lung, kidney and thyroid gland from animals of the intermediate dose groups,
- gross abnormalities from all animals.

For all unscheduled sacrificed or dead animals on study, the cause of death was determined when it was possible.
Initial examinations were performed by the Study Pathologist in Histopathology. Following the initial examination, an in-house review pathologist, undertook an independent « peer-review » of representative slides and diagnoses according to standardized operating procedures. The diagnoses presented in this report represent the consensus opinion of the two pathologists.
Histopathological examinations from final sacrificed animals (105 weeks of treatment) were performed on all organs and tissues embedded including gross abnormalities in all animals from all groups including decedents.
For all unscheduled sacrificed or dead animals on study, the cause of death was determined when it was possible.
Initial examinations were performed by the Study Pathologist in Histopathology. Following the initial examination, an external review pathologist ([redacted], U.K) undertook an independent « peer-review » of representative slides and diagnoses according to standardized operating procedures. The diagnoses presented in this report represent the consensus opinion of the two pathologists.

II. Results and discussion

A. Observations

1. Clinical signs

At 2000 ppm in males, soiled fur, hyper-reactivity to external stimuli and resistance to handling were observed with slightly higher incidences than controls, during the first year of treatment. In females, higher incidence of hair loss was observed at 2000 ppm and to a lesser extends at 400 ppm compared to controls. Soiled fur was also observed with slightly higher incidences at 2000 ppm and 400 ppm compared to controls. However, the localization was not always the same (anogenital region, mouth or nose) and there was no dose-relationship. Therefore this was considered not to be treatment-related.

Table 5.5.2-02: Incidence of treatment-related clinical signs during the first year of treatment (animals allocated to the chronic and carcinogenicity phases)

Sex	Male				Female			
Dosage level of BYI 02960(ppm)	0	80	400	2000	0	80	400	2000
Group size	70	70	70	70	70	70	70	70
Soiled fur	5 (7.1%)	6 (8.6%)	5 (7.1%)	9 (12.9%)	2 (2.9%)	0 (-)	6 (8.6%)	6 (8.6%)
Hair loss	nc	nc	nc	nc	8 (11.4%)	3 (4.3%)	13 (18.6%)	22 (31.4%)
Hyper-reactivity to external stimuli	1 (1.4%)	0 (-)	1 (1.4%)	5 (7.1%)	nc	nc	nc	nc
Resistance to handling	4 (5.7%)	3 (4.3%)	6 (8.6%)	11 (15.8%)	nc	nc	nc	nc

nc : not concerned or no relevant changes

During the second year of treatment, the only treatment-related clinical signs were hair loss and soiled fur which were observed with slightly higher incidences in males at 2000 ppm compared to controls. No treatment-related clinical signs were observed in males at 400 ppm and 80 ppm or in females at any dose levels.

Table 5.5.2-03: Incidence of treatment-related clinical signs during the second year of treatment (animals allocated to the carcinogenicity phases)

Sex	Male				Female			
Dosage level of BYI 02960 (ppm)	0	80	400	2000	0	80	400	**2000**
Group size	58	59	60	60	58	59	56	**60**
Hair loss	1 (1.7%)	2 (3.4%)	2 (3.3%)	4 (6.7%)	nc	nc	nc	**nc**
Soiled fur	12 (20.7%)	10 (16.9%)	11 (18.3%)	19 (31.7%)	nc	nc	nc	**nc**

nc : not concerned or no relevant changes

2. Mortality

During the first year of treatment, mortality was comparable within groups in both sexes with no effect of BYI 02960 administration. Mortality ranged from 10.0% to 12.9% and from 8.6% to 11.4% in males and females, respectively.

Table 5.5.2-04: Mortality incidence after 53 weeks of treatment (animals allocated to the chronic and carcinogenicity phases)

Sex	Male				Female			
Dosage level of BYI 02960 (ppm)	0	80	400	2000	0	80	400	2000
Group size	70	70	70	70	70	70	70	70
Mortality (% mortality)	9 (12.9)	9 (12.9)	7 (10.0)	7 (10.0)	8 (11.4)	8 (11.4)	11 (15.7)	6 (8.6)

At the end of the study, mortality was comparable across the groups in males (ranging from 50% to 65%). In females, a lower mortality was observed at 2000 ppm compared to the control group (23% *vs.* 52%).

Table 5.5.2-05: Mortality incidence after 105 weeks of treatment (animals allocated to the carcinogenicity phases)

Sex	Male				Female			
Dosage level of BYI 02960 (ppm)	0	80	400	2000	0	80	400	2000
Group size	60	60	60	60	60	60	60	60
Accidental trauma	-	1	-	-	-	1	1	-
Died during anesthesia	-	-	-	-	1	1	1	1
Killed for humane reason	16	13	16	7	17	18	18	11
Found dead	19	25	14	26	13	10	8	2
Mortality (% mortality)	35 (58)	39 (65)	30 (50)	33 (55)	31 (52)	30 (50)	28 (47)	14 (23)

3. Neurological examinations

Not evaluated in this study.

B. Body weight

At 2000 ppm in males, the mean cumulative body weight gain was significantly lower than controls during the first week of treatment (- 27%, p≤0.01) and over the first three months of the study (- 13%, p≤0.01). Thereafter, mean cumulative body weight gain was comparable to controls. The mean body weight was lower compared to controls from Week 1 to Week 54 (statistically significant in most occasions). Thereafter the mean body weight was comparable to controls. In females, mean body weight and cumulative body weight gains were lower compared to controls throughout the study (- 5% to - 17%, p≤0.01 or 0.001 for body weight and - 18% to - 62%; p ≤0.001, 0.01 or 0.05 for cumulative body weight gains). Overall, the mean cumulative body weight gain was decreased by 23% when compared to controls at the end of the study, the mean body weight was 13% lower.

At 400 ppm, mean cumulative body weight gain was statistically significantly lower than in controls during the first week of treatment for males (- 6%, p ≤0.05) and females (-12%, p ≤0.05) and was comparable to control thereafter. Mean body weight was not affected throughout the study.

At 80 ppm, there was no treatment-related effect on body weight parameters in either sex.

Table 5.5.2-06: Mean body weight (BW) and cumulative body weight gains (BWG) (g)

BYI 02960 dosage level (ppm)	0	80	400	2000
Male				
Initial BW (Day 1) (%C)	**237**	**237 (100)**	**237 (100)**	**237 (100)**
BW Week 2 (Day 8) (%C)	296	295 (100)	293 (99)	280 ** (95)
BW Week 14 (Day 92) (%C)	544	537 (99)	540 (99)	506 ** (93)
BW Week 26 (Day 176) (%C)	623	612 (98)	615 (99)	585 ** (94)
BW Week 54 (Day 372) (%C)	714	699 (98)	712 (100)	680 * (95)
BW Week 78 (Day 540) (%C)	749	730 (97)	752 (100)	721 (96)
BW Week 106 (Day 736) (%C)	**653**	**623 (95)**	**655 (100)**	**616 (94)**
BWG Weeks 1-2 (Days 1 to 8) (%C)	**59**	**58 (98)**	**56 * (94)**	**43 ** (73)**
BWG Weeks 1-14 (Days 1 to 92) (%C)	308	301 (98)	303 (98)	269 ** (87)
BWG Weeks 14-26 (Days 92 to 176) (%C)	78	75 (96)	75 (96)	80 (103)
BWG Weeks 26-54 (Days 176 to 372) (%C)	89	90 (101)	95 (107)	93 (104)
BWG Weeks 54-78 (Days 372 to 540) (%C)	31	42 (135)	39 (126)	39 (126)
BWG Weeks 78-106 (Days 540 to 736) (%C)	- 91	-84 (nc)	-82 (nc)	-113 (nc)
Overall BWG Weeks 1-106 (Days 1 to 736) (%C)	416	392 (94)	420 (101)	379 (91)
BYI 02960 dosage level (ppm)	0	80	400	2000
Female				
Initial BW (Day 1) (%C)	**167**	**168 (100)**	**168 (100)**	**167 (100)**
BW Week 2 (Day 8) (%C)	192	191 (100)	190 (99)	181 ** (95)
BW Week 14 (Day 92) (%C)	290	288 (99)	289 (100)	267 ** (92)
BW Week 26 (Day 176) (%C)	319	313 (98)	316 (99)	289 *** (91)
BW Week 54 (Day 372) (%C)	364	354 (97)	355 (97)	317 ** (87)
BW Week 78 (Day 540) (%C)	427	430 (102)	417 (99)	349 ** (83)
BW Week 106 (Day 736) (%C)	**421**	**437 (104)**	**444 (105)**	**364 ** (87)**
BWG Weeks 1-2 (Days 1 to 8) (%C)	**25**	**23 (92)**	**22 * (88)**	**15 ** (60)**
BWG Weeks 1-14 (Days 1 to 92) (%C)	122	120 (98)	122 (100)	100 ** (82)
BWG Weeks 14-26 (Days 92 to 176) (%C)	29	26 (90)	27 (93)	22 ** (76)
BWG Weeks 26-54 (Days 176 to 372) (%C)	48	39 (81)	37 (77)	28 *** (58)
BWG Weeks 54-78 (Days 372 to 540) (%C)	59	76 (129)	62 (105)	34 ** (58)
BWG Weeks 78-106 (Days 540 to 736) (%C)	39	21 (54)	35 (90)	15 * (38)
Overall BWG Weeks 1-106 (Days 1 to 736) (%C)	**256**	**271 (106)**	**275 (107)**	**198 *** (77)**

%C : % vs control C : control nc : not calculated
* : Statistically different ($p \leq 0.05$) from the control
** : Statistically different ($p \leq 0.01$) from the control
*** : Statistically different ($p \leq 0.001$) from the control

C. Food consumption and compound intake

1. Food consumption

Overall mean food consumption was considered to be comparable to controls in males at all dose levels with the exception of the first few days of treatment at 2000 ppm where mean consumption was slightly lower than control.

At 2000 ppm in females, mean food consumption was also slightly lower than controls during the first days of treatment (- 15% on study day 5) and was marginally lower than control in many occasions throughout the study. At 400 and 80 ppm, mean food consumption was considered to be similar to controls throughout the study.

Table 5.5.2-07: Group mean food consumption (g/animal/day)

BYI 02960 dosage level (ppm)	0	80	400	2000
Male				
Week period 1 to 13 (% C)	27.2	27.0 (99)	27.1 (100)	26.2 (96)
Week period 14 to 26 (% C)	25.5	25.4 (100)	25.8 (101)	25.0 (98)
Week period 27 to 52 (% C)	25.9	25.2 (97)	25.9 (100)	25.6 (99)
Week period 53 to 78 (% C)	24.1	24.2 (100)	24.3 (101)	24.1 (100)
Week period 79 to 104 (% C)	22.7	22.2 (98)	22.6 (100)	21.9 (96)
Female				
Week period 1 to 13 (% C)	19.8	19.8 (100)	19.7 (99)	19.8 (100)
Week period 14 to 26 (% C)	18.7	18.5 (99)	18.6 (99)	18.7 (100)
Week period 27 to 52 (% C)	19.5	18.8 (96)	18.7 (96)	18.1 (93)
Week period 53 to 78 (% C)	19.5	19.5 (100)	19.5 (100)	17.7 (91)
Week period 79 to 104 (% C)	19.9	20.7 (104)	20.7 (104)	18.5 (93)

2. Achieved dosages

The mean achieved dosage intake per group was as follows:

Table 5.5.2-08: Mean achieved dietary intake of BYI 02960 (mg/kg/day)

Sex	Male				Female			
Dosage level (ppm)	0	80	400	2000	0	80	400	2000
Week period 1 to 13	-	5.01	25.1	129		6.33	31.7	170
Week period 1 to 52	-	3.68	18.5	95.1	-	5.08	25.3	136
Week period 1 to 105	-	3.16	15.8	80.8	-	4.48	22.5	120

3. Food efficiency

Not evaluated in this study.

D. Ophthalmological examination

At the end of the first year of treatment, a slightly increased incidence of lens opacity was observed in females at 2000 ppm. It was recorded in 10/70 females compared to 3/69 control females. No treatment-related ophthalmological findings was noted at any dose level in males or at the mid and low dose levels in females after 1 year of treatment.

Table 5.5.2-9: Incidence of treatment-related ophthalmological findings noted at the 1 year examination (animals allocated to the chronic and carcinogenicity phases)

Sex	Male		Female	
Dosage level of BYI 02960 (ppm)	0	2000	0	2000
Group size	68	70	69	70
Lens opacity	2 (2.9%)	2 (2.9%)	3 (4.3%)	10 (14.3%)

At the end of the second year of treatment, the following treatment-related ophthalmological observations were made.

Table 5.5.2-10: Incidence of treatment-related ophthalmological findings noted at the second year examination (animals allocated to the carcinogenicity phase)

Sex	Male				Female			
Dose level of BYI 02960 (ppm)	0	80	400	2000	0	80	400	2000
Group size	26	24	31	31	30	32	33	48
Iris mydriasis	3 (11.5%)	2 (8.3%)	3 (9.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (8.3%)
Lens opacity HCD - Mean % (Min-Max)	23 (88%)	22 (92%)	29 (94%)	29 (94%)	15 (50%)	22 (69%)	27 (82%)	46 (96%)
	92.0% (81.4 - 100.0)							
Retina, fundus abnormal color, pale	0 (0%)	1 (4.2%)	1 (3.2%)	1 (3.2%)	0 (0%)	0 (0%)	0 (0%)	3 (6.3%)

HCD : Historical Control Data

No treatment-related ophthalmological findings were observed at any dose level in males.
At 2000 ppm in females, iris mydriasis was noted in 4/48 females (0/30 in controls) and an abnormal pale color of fundus of the retina was noted in 3/48 females (0/30 in controls). In addition, higher incidences of lens opacity was observed at this dose level (representing 96% compared to 50% in controls) and to a lesser extent at 400 and 80 ppm (82% and 69%, respectively). Lens opacity is a very common finding in rats of this strain and age as shown by in-house historical data over the last 5 years (mean percentage of 92.0% for females). In this study, the value in the control group was particularly low and even clearly outside our historical control data range (81.4 - 100.0%). However values of all treated groups were well within the range of historical control data. Therefore this change was considered to be neither treatment-related nor toxicologically relevant.

Other ophthalmological changes were considered to be chance findings as they occurred in isolation, in a non dose-related manner or at a similar frequency in the control and treated group.

E. Blood analysis

1. Hematology

Effects were observed at 2000 ppm only.
At 2000 ppm in males, mean leucocyte counts were higher than control values at Month 12 (+ 22%, $p \leq 0.01$), 18 (+ 31%, $p \leq 0.01$) and 24 (+ 63%, $p \leq 0.05$). This variation was associated with higher mean

absolute lymphocyte count at Month 12 (+ 19%, p ≤0.01) and 18 (+ 32%, p ≤0.01) and with higher mean absolute neutrophil count at Month 12 (+ 36%, p ≤0.05) and 24 (+ 143%, p ≤0.05).

At 2000 ppm in females, throughout the first year of treatment, statistically significant differences were noted in some erythrocyte parameters. Mean hemoglobin concentration and/or mean corpuscular volume were lower; as a consequence mean hematocrit and/or mean corpuscular hemoglobin were lower too. These variations were very slight (≤ - 4%, relative to the controls).
In this group, mean platelet counts were slightly higher (≤ + 20%, relative to the controls) during the first year and mean total leucocyte count and mean absolute lymphocyte count were slightly higher at Month 4 (+ 31% and + 34% respectively, relative to the controls) and at Month 6 (+ 34% and + 42% respectively, relative to the controls). These changes were considered not to be toxicologically relevant in view of their low magnitude and their transient occurrence.

The few other differences observed, even if statistically significant, were considered to be incidental and not treatment-related.

2. Clinical chemistry
Effects were observed at 2000 ppm only.
At 2000 ppm, throughout the study, slightly lower mean total bilirubin concentrations were noted in both sexes. However, lower total bilirubin concentrations are considered not to be adverse effects of the test substance as they do not represent any functional impairment in the test organism. The differences observed at 400 and 80 ppm were considered not to be relevant in the absence of consistency during the study.

At 2000 ppm, throughout the study, slightly lower mean total bilirubin concentrations were noted in both sexes. However, lower total bilirubin concentrations are considered not to be adverse effects of the test substance as they do not represent any functional impairment in the test organism. The differences observed at 400 and 80 ppm were considered not to be relevant in the absence of consistency during the study.

Table 5.5.2-11: Total bilirubin concentration (µmol/L) - Mean standard ± deviation (% change when compared to controls)

Dose level of BYI 02960 (ppm)	control	80	400	2000
Male				
Month 4	1.21 ± 0.510[a]	1.23 ± 0.277 (+ 2%)	1.10 ± 0.316 (- 9%)	0.72 ± 0.334*** (-4 0%)
Month 6	1.60 ± 0.383	1.64 ± 0.536 (+ 3%)	1.42 ± 0.378 (- 11%)	1.05 ± 0.254*** (- 34%)
Month 12	1.75 ± 0.494	1.77 ± 0.426 (+ 1%)	1.45 ± 0.405 (- 17%)	1.15±0.560** (-34%)
Month 18	1.66 ± 0.259	1.88 ± 0.767 (+13%)	1.71 ± 0.593 (+ 3%)	1.17 ± 0.302 (- 30%)
Month 24	2.11 ± 0.743	1.81 ± 0.729 (- 14%)	1.64 ± 0.438 (- 22%)	1.63 ± 1.763 (- 23%)
Female				
Month 4	2.08 ± 0.651	2.18 ± 0.507 (+ 5%)	1.75 ± 0.517 (- 16%)	1.13 ± 0.339** (- 46%)
Month 6	2.45 ± 0.604	2.56 ± 0.494 (+ 4%)	2.00 ± 0.407* (- 18%)	1.34 ±0 .347** (- 45%)
Month 12	2.69 ± 0.761	2.80 ± 0.710 (+ 4%)	2.93 ± 0.945 (+ 9%)	1.61 ±0.448*** (-40%)
Month 18	2.47 ± 0.917	1.81 ± 0.857 (- 27%)	1.88 ± 1.104 (-24%)	1.34 ± 0.613 (- 46%)
Month 24	2.01 ± 0.711	1.82 ± 0.588 (- 9%)	1.95 ± 0.712 (- 3%)	1.24 ± 0.363* (- 38%)

*** : $p \leq 0.001$ ** : $p \leq 0.01$ * : $p \leq 0.05$

[a] : mean and standard deviation were calculated excluding a very high value (149.4µmol/L) for animal T1M1177 which was considered to have a transient icterus. % changes in the treated groups were calculated with this new mean value, statistical analyses were applied without exclusion.

At 2000 ppm, throughout the study, slightly higher mean total cholesterol concentrations were observed in females. No relevant change was noted in males.

Table 5.5.2-12: Total cholesterol concentration (mmol/L) - Mean standard ± deviation (% change when compared to controls)

Dose level of BYI 02960 (ppm)	control	80	400	2000
Female				
Month 4	1.680 ± 0.2990	1.687 ± 0.3253 (0%)	1.648 ± 0.3807 (- 2%)	2.164 ± 0.3220** (+ 29%)
Month 6	1.713 ± 0.2973	1.790 ± 0.2800 (+ 4%)	1.804 ± 0.3153 (+ 5%)	2.316 ± 0.4057** (+ 35%)
Month 12	1.923 ± 0.3674	1.921 ± 0.4184 (0%)	2.037 ± 0.3878 (+ 6%)	2.393 ± 0.3235** (+ 24%)
Month 18	2.049 ± 0.4321	2.010 ± 0.3739 (- 2%)	2.182 ± 0.5346 (+ 6%)	2.380 ± 0.5004 (+ 16%)
Month 24	1.936 ± 0.3417	2.281 ± 0.3718 (+ 18%)	2.136 ± 0.3439 (+ 10%)	2.352 ± 0.6305 (+ 21%)

** : $p \leq 0.01$

When compared to the control groups, at 2000 ppm, slightly lower mean glucose concentrations were observed at Month 4 in both sexes (- 23%, $p \leq 0.001$ in males and - 18%, $p \leq 0.01$ in females) and at Month 6 in males only (-16%, $p \leq 0.05$). The variations observed thereafter were considered not relevant.
In females at 2000 ppm, statistically significant differences were seen at several sampling periods for inorganic phosphorus concentration, however there was no consistency throughout the study. Therefore this slight change was considered to be not relevant.

F. Urinalysis

No relevant treatment-related change was observed.
When compared to the controls, a tendency towards lower pH values was noted throughout the study at 2000 ppm in males only. However in view of the individual data variation, these slight differences were considered not to be biologically relevant.
The other differences were few and considered to be incidental.

G. Bioanalytical evaluation

Plasmatic concentrations of BYI 02960 after 12 and 24 months were as follows:

Table 5.5.2-13: BYI 02960 plasmatic concentration (mg/L) - Mean standard ± deviation

Dose level of BYI 02960 (ppm)	control	80	400	2000
Male				
Month 12	<LLOQ	1.29 ± 0.24	5.17 ± 0.38	18.8 ± 0.98
Month 24	<LLOQ	1.51 ± 0.10	7.19 ± 1.18	21.4 ± 4.47
Female				
Month 12	<LLOQ	1.36 ± 0.18	7.84 ± 1.86	33.7 ± 3.47
Month 24	<LLOQ	1.06 ± 0.50	8.20 ± 1.35	30.3 ± 3.80

<LLOQ: below the lower limit of quantitation of 25 µg/L

As expected, plasmatic concentrations of BYI 02960 increased with the dietary dose levels.

H. Sacrifice and pathology

1. Terminal body weight and organ weights -12-month chronic phase

At 2000 ppm in females mean terminal body weight was statistically significantly lower (- 20%, $p \leq 0.001$), when compared to controls.
A slightly lower mean terminal body weight was observed in females at 400 ppm and 80 ppm (respectively - 7% and - 12%, not statistically significant) when compared to the controls. In the absence of dose-relationship and without any correlated adverse macroscopic or microscopic at these dose levels, these variations were considered not to be treatment-related.
At 2000 ppm, in both sexes, mean liver to body weight ratios were statistically significantly higher when compared to controls and were considered to be treatment-related (associated with microscopic hepatocellular hypertrophy).

Table 5.5.2-14: Mean liver weight ± SD at 12-month scheduled sacrifice (% change when compared to controls)

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Absolute liver weight (g)	13.421 ± 1.7893	13.222 ± 1.5469 (- 1%)	13.137 ± 1.7780 (- 2%)	14.856 ± 1.8431 (+ 11%)	7.854 ± 1.3550	7.182 ± 1.1995 (- 9%)	7.245 ± 1.2346 (- 8%)	7.222 ± 0.4811 (- 8%)
Liver to body weight ratio (%)	1.993 ± 0.1374	1.970 ± 0.1408 (- 1%)	1.946 ± 0.1838 (- 2%)	2.285** ± 0.1736 (+ 15%)	2.135 ± 0.1449	2.194 ± 0.2280 (+ 3%)	2.101 ± 0.1524 (- 2%)	2.444** ± 0.1080 (+ 14%)
Liver to brain weight ratio (%)	587.888 ± 70.5766	573.101 ± 74.3182 (- 3%)	572.059 ± 78.7533 (- 3%)	670.435 ± 80.8317 (+ 14%)	373.068 ± 66.9021	344.168 ± 59.4331 (- 8%)	364.246 ± 72.3178 (- 2%)	346.880 ± 18.2160 (- 7%)

** : $p \leq 0.01$

2. Terminal body weight and organ weights - 24-month carcinogenicity phase

At 2000 ppm in females mean terminal body weight was statistically significantly lower (- 14%, $p \leq 0.001$), when compared to controls.

A slightly lower mean terminal body weight was observed in males at 2000 ppm (- 7%, not statistically significant) when compared to the controls.

At 2000 ppm in both sexes, mean liver to body weight ratios were statistically significantly higher when compared to controls and were judged to be treatment-related (associated with microscopic hepatocellular hypertrophy).

Table 5.5.2-15: Mean liver weight ± SD at 24-month scheduled sacrifice (% change when compared to controls)

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Absolute liver weight (g)	12.401 ± 1.9940	12.065 ± 1.8556 (-3%)	11.971 ± 1.8261 (- 3%)	12.915 ± 1.4947 (+ 4%)	8.878 ± 1.7052	9.246 ± 1.6570 (+ 4%)	9.406 ± 2.1925 (+ 6%)	8.308 ± 1.3457 (- 6%)
Liver to body weight ratio (%)	1.971 ± 0.2142	2.008 ± 0.2758 (+ 2%)	1.941 ± 0.1859 (- 2%)	2.215** ± 0.3085 (+ 12%)	2.242 ± 0.2658	2.291 ± 0.3077 (+ 2%)	2.278 ± 0.3116 (+ 2%)	2.458** ± 0.2783 (+ 10%)
Liver to brain weight ratio (%)	530.945 ± 90.8128	518.885 ± 81.6223 (- 2%)	514.364 ± 79.8667 (- 3%)	546.361 ± 64.5187 (+ 3%)	418.821 ± 79.1927	440.047 ± 79.7220 (+ 5%)	449.204 ± 105.7920 (+ 7%)	392.895 ± 67.0007 (- 6%)

** : $p \leq 0.01$

3. Gross pathology - 12-month chronic phase

Unscheduled death

Five animals died before the end of the study.

One male from group 2 was found dead on study Day 312, with a pale appearance, a loss of tail secondary to cannibalism, red foci on the right ventricle of the heart and an autolysis.

One male from group 3 died during anesthesia on study Day 180, with red foci on lungs and thymus.

One female from group 2 died during anesthesia on study Day 361, with dilatation of left uterine horn uterus, an ovarian cyst and a general red appearance.
One female from group 3 died during anesthesia on study Day 98, with red foci on thymus.
Another female from group 3 died during anesthesia on study Day 361, with red foci on thymus an ovarian cyst, red mottled lungs and a traumatic lesion in the liver.

Terminal sacrifice
At 2000 ppm in males, enlarged and pale liver were noted (3/10) and considered to be treatment-related (associated with microscopic findings).

Table 5.5.2-16: Incidence of macroscopic changes in the liver, scheduled sacrifice, chronic phase.

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Enlarged	0/10	0/9	0/9	3/10	1/10	1/9	0/8	0/10
Pale	0/10	1/9	0/9	3/10	1/10	1/9	1/8	0/10

4. Gross pathology - 24-month carcinogenicity phase
Unscheduled death
Two hundred forty animals died before the end of the study.
At 2000 ppm, white foci in the lung were noted (6/14 females): this increased incidence was considered to be treatment-related (associated with microscopic findings).

Table 5.5.2-17: Incidence of macroscopic changes in the lung, unscheduled sacrifice, carcinogenicity phase

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Focus(i), white	4/35	8/39	5/30	7/33	2/31	2/30	2/28	6/14

All other changes were considered as incidental and not treatment-related.

Terminal sacrifice
At 2000 ppm, white foci in the lung were noted (21/46 females): this increased incidence was considered to be treatment-related (associated with microscopic findings).

Table 5.5.2-18: Incidence of mascroscopic changes in the lung, scheduled sacrifice, carcinogenicity phase

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Focus(i), white	5/25	4/21	4/30	5/27	2/29	10/30	5/32	21/46

At 2000 ppm, higher incidence of cysts in the ovary were noted (18/46 females), when compared to controls (8/29).
Microscopically there was no difference in terms of percentages in ovarian cysts between control group (18/29; 62%) and 2000 ppm group (31/46, 67%). So this change was considered not to be treatment-related.

5. Microscopic pathology - 12-month chronic phase
Terminal sacrifice and unscheduled animals

Non-neoplastic findings
In the liver, relevant higher incidences of eosinophilic and tigroid foci of altered hepatocytes were observed in males at 2000 ppm. Centrilobular hypertrophy was observed in both sexes at 2000 ppm and also at 400 ppm in 2/10 males (dose-related effect). However in the absence of any increase of incidences of foci of alteration or hepatocellular macrovacuolation, this change at 400 ppm was considered not to be an adverse effect. Centrilobular hepatocellular macrovacuolation and lower incidences of periportal hepatocellular vacuolation were noted in both sexes at 2000 ppm.

Table 5.5.2-19: Incidence and severity of microscopic changes in the liver, all animals of the chronic phase

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Number of examined animals	10	10	10	10	10	10	10	10
Eosinophilic focus(i) of hepatocellular alteration								
Minimal	2	6	3	8	0	0	1	1
Total	**2**	**6**	**3**	**8**	**0**	**0**	**1**	**1**
Tigroid focus(i) of hepatocellular alteration								
Minimal	1	1	0	3	3	2	1	1
Total	**1**	**1**	**0**	**3**	**3**	**2**	**1**	**1**
Hepatocellular hypertrophy: centrilobular								
Minimal	0	0	2	7	0	0	0	5
Slight	0	0	0	3	0	0	0	0
Total	**0**	**0**	**2**	**10**	**0**	**0**	**0**	**5**
Hepatocellular macrovacuolation: centrilobular: diffuse								
Minimal	0	2	1	6	0	0	0	2
Slight	0	0	0	4	0	0	0	0
Total	**0**	**2**	**1**	**10**	**0**	**0**	**0**	**2**
Hepatocellular vacuolation: mainly periportal: diffuse								
Minimal	5	6	3	4	6	8	7	4
Slight	1	1	2	0	2	0	3	0
Moderate	0	0	1	0	0	0	0	0
Total	**6**	**7**	**6**	**4**	**8**	**8**	**10**	**4**

In the thyroid gland, colloid alteration was found to be increased in incidence and severity in both sexes at 2000 ppm and in males at 400 ppm.

Table 5.5.2-20: Incidence and severity of microscopic changes in the thyroid gland, all animals of the chronic phase (cont'd)

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Number of examined animals	10	10	10	10	10	10	10	10
Colloid alteration								
Minimal	2	1	6	3	0	0	0	3
Slight	0	1	1	3	0	0	0	0
Moderate	0	1	0	0	0	0	0	0
Marked	0	1	0	0	0	0	0	0
Total	**2**	**4**	**7**	**6**	**0**	**0**	**0**	**3**

Neoplastic findings

No neoplastic change was noted during the chronic phase.

6. Microscopic pathology - 24-month carcinogenicity phase

Terminal sacrifice and unscheduled animals

Non-neoplastic findings:

In the liver, higher incidences of eosinophilic, mixed and tigroid foci of altered hepatocytes were observed in males at 2000 ppm. Centrilobular hypertrophy and higher incidences of centrilobular hepatocellular macrovacuolation were observed in both sexes at 2000 ppm. Higher incidences of hepatocellular brown pigments/brown pigments in Kupffer cells, higher incidence of interstitial mononuclear cell infiltrate and lower incidence of periportal hepatocellular macrovacuolation were noted in females at 2000 ppm.

No adverse effect was noted on both sexes at 400 ppm, there was only minimal centrilobular hypertrophy in 6 male animals without any increase of foci of alteration (preneoplastic finding) or hepatocellular macrovacuolation (sign of hepatocellular metabolic disturbance).

Table 5.5.2-21: Incidence and severity of microscopic changes in the liver, all animals of the carcinogenicity phase

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Number of examined animals	60	60	60	60	60	60	60	60
Eosinophilic focus(i) of hepatocellular alteration								
Minimal	22	21	19	28	14	9	8	16
Slight	4	6	9	9	2	3	1	6
Moderate	1	0	2	7	0	0	1	1
Marked	0	0	0	1	0	0	0	0
Total	**27****	**27**	**30**	**45****	**16**	**12**	**10**	**23**
Tigroid focus(i) of hepatocellular alteration								
Minimal	29	28	32	42	30	30	24	36
Slight	5	8	7	3	5	7	5	5
Moderate	0	0	0	0	1	0	1	0
Total	**34**	**36**	**39**	**45**	**36**	**37**	**30**	**41**
Mixed focus(i) of hepatocellular alteration								
Minimal	0	0	0	3	0	0	0	0
Total	**0***	**0**	**0**	**3**	**0**	**0**	**0**	**0**
Hepatocellular hypertrophy: centrilobular								
Minimal	0	0	6	23	0	0	0	27
Slight	0	0	0	2	0	0	0	1
Total	**0****	**0**	**6***	**25****	**0****	**0**	**0**	**28****
Hepatocellular macrovacuolation: centrilobular: diffus								
Minimal	3	4	3	18	1	0	0	19
Slight	0	0	1	6	0	1	0	5
Moderate	0	1	0	0	1	0	0	0
Total	**3****	**5**	**4**	**24****	**2****	**1**	**0**	**24****
Hepatocellular macrovacuolation: periportal: diffuse								
Minimal	5	4	2	2	18	22	12	11
Slight	2	3	1	4	8	11	13	2
Moderate	2	1	1	0	2	3	1	0
Marked	0	0	1	1	0	0	0	0
Total	**9**	**8**	**5**	**7**	**28****	**36**	**26**	**13****
Hepatocellular brown pigment: focal								
Minimal	0	0	0	0	1	0	1	13
Slight	0	0	0	0	0	0	0	3
Total	**0**	**0**	**0**	**0**	**1****	**0**	**1**	**16****

* : Significant ($p \leq 0.05$) **: Significant ($p \leq 0.01$)

Table 5.5.2-21: Incidence and severity of microscopic changes in the liver, all animals of the carcinogenicity phase (cont'd)

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Number of examined animals	60	60	60	60	60	60	60	60
Accumulation of brown pigment in Kupffer cells								
Minimal	8	11	10	9	10	9	14	18
Slight	2	5	8	4	4	4	1	8
Moderate	0	0	0	1	1	1	1	1
Total	**10**	**16**	**18**	**14**	**15***	**14**	**16**	**27***
Interstitial mononuclear cell infiltrate: focal								
Minimal	20	19	21	24	22	28	29	35
Slight	1	0	1	2	1	4	0	1
Moderate	0	0	0	0	1	0	0	0
Total	**21**	**19**	**22**	**26**	**24**	**32**	**29**	**36***

*: Significant ($p \leq 0.05$) **: Significant ($p \leq 0.01$)

In the lung, higher incidences of foamy macrophages, chronic interstitial inflammation and perivascular inflammation were observed in females at 2000 ppm.

Table 5.5.2-22: Incidence and severity of microscopic changes in the lung, all animals of the carcinogenicity phase

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Number of examined animals	60	60	60	60	60	60	60	60
Alveolar foamy macrophages: focal								
Minimal	18	24	22	25	27	18	11	25
Slight	10	12	8	7	2	11	16	18
Moderate	2	4	4	3	1	0	2	6
Marked	0	0	0	0	0	0	0	1
Total	**30**	**40***	**34**	**35**	**30****	**29**	**19***	**50****
Chronic interstitial inflammation: focal								
Minimal	1	4	4	3	2	3	5	10
Slight	0	1	0	0	0	0	0	0
Moderate	0	0	1	0	0	0	0	0
Total	**1**	**5**	**5**	**3**	**2***	**3**	**5**	**10***
Perivascular inflammation: focal								
Minimal	23	20	30	20	17	22	17	35
Slight	1	3	2	1	0	1	0	1
Total	**24**	**23**	**32**	**21**	**17****	**23**	**17**	**36****

* : Significant ($p \leq 0.05$) ** : Significant ($p \leq 0.01$)

In the thyroid gland relevant increased incidences of colloid alteration were noted in males at 2000 ppm and 400 ppm. The increased incidence of colloid alteration at 400 ppm is not considered to be an adverse effect per se: it is naturally observed in controls aging rats and reflects a normal physiologic process associated with the unique rapid turnover of colloid. It was not associated with relevant follicular hypertrophy at this dose.

Relevant higher incidences of follicular cell pigments were noted in both sexes at 2000 ppm.

Table 5.5.2-23: Incidence and severity of microscopic changes in the thyroid gland, all animals of the carcinogenicity phase

Sex	Male				Female			
BYI 02960 Dose-level (ppm)	0	80	400	2000	0	80	400	2000
Number of examined animals	60	60	60	60	60	60	60	60
Colloid alteration								
Minimal	14	12	28	18	11	10	10	11
Slight	7	9	9	18	1	3	0	4
Moderate	0	1	1	4	0	1	0	0
Marked	0	0	0	0	1	0	0	0
Total	21**	22	38**	40**	13	14	10	15
Follicular cell hypertrophy, diffuse								
Minimal	1	0	1	3	0	1	0	3
Total	**1**	**0**	**1**	**3**	**0**	**1**	**0**	**3**
Brown pigments: follicular cells								
Minimal	16	10	18	22	8	7	5	17
Slight	1	0	1	1	0	0	0	0
Moderate	0	0	1	0	0	0	0	0
Total	**17**	**10**	**20**	**23**	**8**	**7**	**5**	**17**

** : Significant ($p \leq 0.01$)

Neoplastic findings

No neoplastic change was noted during the carcinogenicity phase.

III. Conclusions

In conclusion, no relevant treatment-related neoplastic changes were observed at any dose level tested in either sex.

The No Observed Adverse Effect Level (NOAEL) over a 12-month period of dietary administration with BYI 02960 to the Wistar rat was 400 ppm in both sexes (equivalent to 18.5 mg/kg body weight/day in males and 25.3 mg/kg body weight/day in females).

The No Observed Adverse Effect Level (NOAEL) over a 24-month period of dietary administration with BYI 02960 to the Wistar rat was 400 ppm in both sexes (equivalent to 15.8 mg/kg/day weight/day in males and 22.5 mg/kg body weight/day in females).

KIIA 5.5.3 - Carcinogenicity study in the mouse

Report:	**KIIA 5.5.3/01 [redacted] P.; 2012**
Title:	BYI 02960, Carcinogenicity study in the C57BL/6J mouse by dietary administration
Report No & Document No	SA 08338 M-425975-01-1
Guidelines:	OECD 451 (1981); EEC Directive 88/302/EEC – Annex V - Method B.32. (1987); EPA Health Effects Test Guideline (OPPTS 870.4200; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines
GLP	Yes (certified laboratory)

Executive Summary

Groups of 60 male and 60 female C57BL/6J mice were fed diet containing 0, 70, 300 or 1500 ppm of BYI 02960 (batch number 2009-000239, a beige powder, 96.2% w/w) for 52 weeks. After 52 weeks, 10 males and 10 females from each group allocated to the chronic phase of the study were necropsied at the scheduled interim sacrifice. The remaining 50 animals/sex/group, allocated to the carcinogenicity phase of the study, continued treatment until the scheduled final sacrifice of the study after at least 78 weeks of treatment. The mean intake BYI 02960 over 18 months was 0, 10.0, 43 and 224 mg/kg/day in males and 0, 12.2, 53 and 263 mg/kg/day in females, at 0, 70, 300 and 1500 ppm, respectively). Mortality and clinical signs were checked daily. Additionally, detailed physical examinations including palpation for masses were performed weekly throughout treatment. Body weight and food consumption were measured weekly for the first 13 weeks of the study, then monthly thereafter. Hematology determinations were performed at approximately 12 and 18 months from designated animals (20/sex/group). Where possible, blood smears were prepared from moribund animals just before sacrifice. At scheduled sacrifice, body weights were recorded prior to necropsy. All animals were subjected to necropsy, with selected organs weighed at scheduled interim and final sacrifices. Designated tissues were fixed and those allocated to the carcinogenicity phase were examined microscopically.

There was no treatment-related effect on clinical signs, mortality, at the hematology evaluation, or on an earlier development or increased incidence of tumors in any dose group throughout the study.

<u>At 1500 ppm</u>

Mean body weight was progressively decreased in both sexes throughout the study by up to 7% in males ($p \leq 0.01$ on Weeks 38, 58, 66 and 70) and 8% in females ($p \leq 0.01$ on Week 66), compared to controls. Mean cumulative body weight gain was also significantly lower than controls throughout most study intervals in males (Weeks 1 to 2, 14 to 26 and 54 to 78) and from Week 14 onwards in females (Weeks 14 to 26, 26 to 54 and 54 to 78). Consequently, overall mean cumulative body weight gain between Days 1 to 540 (Weeks 1 to 78) was 19% lower than controls in males and 13% lower than controls in females. Mean food consumption was marginally decreased during the first two weeks of the study in males (- 4% on Week 1 and - 3% on Week 2, not statistically significant) and on several occasions throughout the study in females (average decrease of 3%), compared to controls. The

concentration of BYI 02960 in plasma was 27.4 mg/L in males and 24.8 mg/L in females at Week 52 and 30.3 mg/L in males and 28.7 mg/L in females at Week 78.
At the 12-month interim sacrifice, mean terminal body weight was lower in males (- 6%, not statistically significant), when compared to control animals. Mean absolute kidney weight and mean kidney to brain weight ratio were 13 to 15% lower than controls in males. Mean brain to body weight ratio was 9% higher than controls in males, but this effect was related to the lower mean body weight. At the macroscopic observation, no treatment-related changes were noted in either sex. Histopathology was not performed for the chronic phase animals.

At the 18-month terminal sacrifice of the carcinogenicity phase of the study, mean terminal body weight was lower in males (- 4%, $p \leq 0.01$) and females (- 6%, not statistically significant), when compared to controls. Mean absolute and relative liver weights were 9 to 14% higher than controls in males. Mean liver to body weight ratio was also 8% higher in females, but this effect was related to the lower mean body weight. Mean absolute and relative kidney weights were 10 to 14% lower than controls in males. Mean brain to body weight ratio was slightly higher than controls in both sexes, but this effect was related to the lower mean body weight. At the macroscopic observation, atrophic/small kidneys were noted in 5/42 males. At the microscopic examination, there were only non-neoplastic findings noted in the liver and kidney. In the liver, a higher incidence and severity of diffuse hepatocellular vacuolation (mainly centrilobular) was noted in males (statistically significant), whilst a decreased incidence of diffuse hepatocellular macrovacuolation (mainly periportal) was noted in females (statistically significant). These changes in the liver were considered to be treatment-related but not adverse (no associated degenerative changes). In the kidney, decreased incidence and severity of bilateral basophilic tubules, focal cortical mineralization and corticoepithelial vacuolation were noted in males (statistically significant). These changes in the kidney were considered to be treatment-related but not adverse (lower incidence and severity of a common finding observed in control animals).

<u>At 300 ppm</u>
No treatment-related changes were noted at this dose level in either sex for any of the parameter evaluated, with the exception of occasional effects on mean body weight parameters and microscopic changes in the liver and kidney of carcinogenicity phase male animals.

Mean body weight was similar to controls throughout the study, except on a few occasions where a slight decrease by up to 3% in males and 5% in females was observed, in comparison to controls. Mean cumulative body weight gain was also slightly lower than controls on a few occasions in both sexes throughout the study, resulting in an overall mean cumulative body weight gain between Days 1 to 540 (Weeks 1 to 78) 6% lower than controls in males and 10% lower than controls in females (not statistically significant). These changes on mean body weight parameters were considered not to be adverse in both sexes, in view of their low magnitude or occasional occurrences. The concentration of BYI 02960 in plasma was 6.05 mg/L in males and 4.01 mg/L in females at Week 52 and 6.58 mg/L in males and 3.90 mg/L in females at Week 78.

At the 18-month terminal sacrifice of the carcinogenicity phase of the study, the only changes were noted at the microscopic examination in males and consisted of a higher incidence and severity of diffuse hepatocellular vacuolation (mainly centrilobular) in the liver (not statistically significant) together with a decreased severity of corticoepithelial vacuolation in the kidney. Both changes were

considered to be treatment-related but not adverse (no associated degenerative changes and lower severity of a common finding observed in control animals, respectively).

At 70 ppm
No treatment-related effects were observed at this dose level. The concentration of BYI 02960 in plasma was 1.29 mg/L in males and 0.94 mg/L in females at Week 52 and 1.45 mg/L in males and 1.03 mg/L in females at Week 78.

In conclusion, dietary administration of BYI 02960 over an 18-month period to the C57BL/6J mouse at dose levels up to 1500 ppm (equivalent to 224 mg/kg/day in males and 263 mg/kg/day in females) did not induce carcinogenic effects.

A dose level of 300 ppm (equivalent to 43 mg/kg/day in males and 53 mg/kg/day in females) was considered to be a No Observed Adverse Effect Level (N.O.A.E.L.) in both sexes over an 18-month period of dietary administration.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Beige powder
Lot/Batch: 2009-000239
Purity: 96.2%
CAS: 951659-40-8
Stability of test compound: Stable in rodent diet at 70 and 2500 ppm for up to 110 days when kept at room temperature

2. Vehicle and /or positive control: None

3. Test animals:
Species: Mouse
Strain: C57BL/6J
Age: 6 weeks approximately
Weight at dosing: 20.59-20.64g for male mean group weight
16.84-17.22g for female mean group weight
Source: [redacted], France
Acclimation period: 14 days
Diet: Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum* except during designated time period
Water: Filtered and softened tap water, *ad libitum*
Housing: Animals were caged individually in suspended stainless steel wire mesh cages. During the first week of acclimatization, the animals were housed by sex in groups of 3.

Environmental conditions: Temperature: 22 ± 2 °C
Humidity: 55 ± 15%
Air changes: Approximately 10 to 15 changes per hour
Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design and methods

1. In life dates

April 08 2009 to November 05, 2010 at [redacted], France.

2. Animal assignment and treatment

On the day of randomization, animals were allocated to dose groups using a computerized randomization procedure that ensured a similar body weight distribution for each sex. The acceptable body weight range for each sex was ± 20% of the mean body weight on the day of randomization. Any animal deemed unsuitable for selection based on weight, clinical findings or health status was not used for the study.

Animals were assigned to the test groups noted in the following table. Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 5.5.3-01: Study design

Test group	Concentration in diet (ppm)	Dose per animal (week period 1 to 80)		Animals assigned/sex	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Main study 78 weeks	Interim sacrifice 52 weeks
1	0	0	0	50	10
2	70	10.0	12.2	50	10
3	300	43	53	50	10
4	1500	224	263	50	10

On Day 7 of the acclimatization phase, each animal was identified by a micro identification implant from Reseaumatique (Bernay, France).

Dose selection

The dose levels were selected based on the results from a previous 90-day dietary study in the mouse, SA 07295, where BYI 02960 was administered in the diet up to 2500 ppm. At 2500 ppm, a lower mean body weight was observed in both sexes throughout the study. The overall cumulative mean body weight over the entire study duration (Days 1 to 92) was lower by 43% in males and by 7% in females. A slight reduction in mean food consumption was observed for females between Study Days 1 and 22, by between 10 and 11%, when compared to controls. Clinical chemistry evaluation revealed a lower mean total cholesterol concentration in both sexes (- 30% and - 24% in males and females, respectively) compared to controls. Higher mean alkaline phosphatase activity was noted in males (+ 38%) whereas mean alanine and aspartate aminotransferase activities were higher in females (+ 106% and +36%, respectively). In both sexes, mean urea concentrations were higher (+ 51% and + 19% in males and

females, respectively) and total protein and albumin concentrations were slightly lower (between - 3% and - 8% in males and females). Mean terminal body weight was statistically significantly lower in males (- 11%) when compared to controls. Mean absolute and relative liver weights were statistically significantly higher in females (+ 12% to 18% when compared to controls). Mean absolute kidney weight and mean kidney to brain weight ratio were statistically significantly lower (- 11%) in males when compared to controls. At macroscopic examination, pale liver was noted in 6/10 females. At microscopic examination, a slight increase in severity of diffuse hepatocellular vacuolation was noted in the liver in both sexes. In the kidney, a loss of the normal multifocal/diffuse cortical epithelial vacuolation was noted in males. At 500 ppm, mean body weight gain/day was marginally reduced in males compared to controls between Study Days 1 and 8 only. At 100 ppm, there were no treatment-related changes.

3. Diet preparation and analysis

The test substance was incorporated into the diet (Certified Rodent Meal A04CP-10 from SAFE) to provide the required dietary concentrations of 70, 300 or 1500 ppm. A small amount of acetone solution was used to facilitate test substance mixture in the diet (acetone evaporated during the mixture process). The test substance formulations were prepared for periods of at least 8 weeks. For control groups, a control formulation was prepared by adding an equivalent volume of acetone into the diet. The diet formulations were stored at ambient temperature until use. The stability of the test substance in the diet had been initially checked in a non-GLP study SA 09014 and was confirmed at 70 ppm in the present study (ranging from 89 to 97% of the nominal concentration) and at 2500 ppm in study SA 08337. Hence, the stability of the test substance at 70 and 2500 ppm in the diet was verified for up to 110 days when kept at room temperature.

Ten formulations were prepared during the study at each concentration. A formulation sequence consisted of one load weighing approximately 50 l to 65 kg.

The homogeneity of the test substance in diet was verified at 70, 300 and 1500 ppm on the first formulation and at 70 and 1500 ppm on the seventh formulation, to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. The homogeneity analyses were within 87 to 98% of the nominal concentration.

The concentration was verified prior to administration to the animals on formulations F1, F3, F7 and F9 for all preparations not checked for homogeneity. The concentration analyses were within 89 to 97% of the nominal concentration.

4. Statistics

Means and standard deviations were calculated for each group and per time period. The Bartlett test was performed to compare the homogeneity of group variances.
If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA), which was followed by Dunnett test (2-sided) if ANOVA indicated significance.

- If the Bartlett test was significant ($p \leq 0.05$) (for body weight change parameters, terminal body weight, absolute and relative organ weight parameters, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin

concentration, %Europhiles, %lymphocytes), group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance.

- If the Bartlett test was significant ($p \leq 0.05$) (for body weight and average food consumption/day parameters), data were transformed using the log transformation. If the Bartlett test on log transformed data was not significant ($p > 0.05$), means were compared using the ANOVA on log transformed data, which was followed by the Dunnett test (2-sided) on log transformed data if ANOVA indicated significance. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided), if the Kruskal-Wallis test indicated significance.
- If the Bartlett test was significant ($p \leq 0.05$) (for haematology parameters such as red blood cell count, platelet count, white blood cell count, neutrophil count, lymphocyte count), data were transformed using the square root transformation. If the Bartlett test on square root transformed data was not significant ($p > 0.05$), means were compared using the ANOVA on square root transformed data, which was followed by the Dunnett test (2-sided) on square root transformed data, if ANOVA indicated significance.
- If the Bartlett test was significant ($p \leq 0.05$) even after square root transformation, group means were compared using the non-parametric Kruskal-Wallis which was followed by the Dunn test (2-sided), if Kruskal-Wallis test indicated significance.

If one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Pristima System version 6.7.0 build 19, an upgraded version of Xybion PathTox NG.

For survival analysis

Adjusted mortality rates were estimated using Kaplan-Meier estimation procedures. Kaplan-Meier estimates were calculated separately for each sex and treatment group. Mortalities which were the result of animals dead at anesthesia, killed following accidents (accidental trauma)or at scheduled sacrifices were considered to be censored observations.

Statistical significance of differences in survival rates between treated and control groups and dose-related trend in survival were assessed using Cox's and Tarone's tests. Probability values presented were two-sided for pair wise comparisons and trend test. Group mortality rates were compared at the 5% and 1% levels of significance. Survival analyses were performed on the carcinogenicity phase.

Clinical signs and macroscopic findings

Selected clinical signs and macroscopic findings were analyzed using the Fisher's exact test (1-sided) for control versus treatment comparisons.

Neoplastic and non-neoplastic findings analyses

When the incidences of the 70 and 300 ppm treated groups were equal to 0, only the 1500 ppm treated group was compared to the control group and no trend test was performed.

Selected lesions were analyzed by Cochran-Armitage method for trend (1-sided) and the Fisher's exact test (1-sided) for control versus treatment comparisons. Cochran-Armitage trend significance is reported when there is existence of any monotone response in the incidence data [that is there is a relationship

between the response and the dose represented by a regression line that is continually increasing (or decreasing), but perhaps not in a straight line].
All tissues missing, autolytic and non-readable, inadequate did not contribute to the analyses.

Group incidences were compared at least at the 5% and 1% levels of significance.
Statistical analyses were conducted using SAS programs (Version 9.2) and LTA.EXE.
Survival analyses and statistical analyses of non-neoplastic and neoplastic lesions were performed on carcinogenicity phase.

C. Methods

1. Clinical signs and mortality
Animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily throughout the study, except on the last necropsy day in error (protocol deviation). Additional detailed physical examinations including palpation for masses were performed at least weekly throughout the study. The nature, onset, severity, duration and reversibility of clinical signs and the onset, location, dimension, appearance, progression and duration of the masses was recorded.

2. Body weight
Each animal was weighed at least weekly during the acclimatization period then weekly for the first 13 weeks of study, approximately every 4 weeks thereafter and prior to necropsy. Body weights recorded prior to necropsy are referred to terminal body weights.

3. Food consumption and compound intake
Food consumption was recorded weekly during the first 13 weeks of treatment, and once approximately every 4 weeks thereafter. The weekly mean achieved dosage intake in mg/kg body weight/day was calculated for Weeks 1 to 13, then 1 week per month thereafter.
The monthly and overall mean achieved dosage intake for the 18 months of treatment were derived from the weekly data.

4. Ophthalmological examination
Not evaluated in the present study. Not required for carcinogenicity studies based on Guideline 870.4200 & OECD 451.

5. Haematology and clinical chemistry
Blood was sampled from Isoflurane anaesthetized animals by puncture of the retro-orbital venous plexus after overnight fasting. Blood was collected in tubes containing EDTA (0.5 mL).
At terminal sacrifice, blood smears were prepared for all animals not sampled for haematology. When possible, a blood smear was prepared for the moribund animals, just before sacrifice.

Haematology
Haematology was performed on all the surviving animals of the interim sacrifice groups and on the first ten surviving animals of the terminal sacrifice groups on Weeks 53 or 54.

Haematology was performed on the first twenty surviving suitable mice of the terminal sacrifice groups prior to necropsy on Weeks 79 or 80. The following parameters were measured using an Advia 120 (Siemens, Eragny, France).: haematocrit, haemoglobin, leukocyte count, erythrocyte count, platelet count, leukocyte differential count, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, mean corpuscular volume.

For all sampled or sacrificed (except on weekends and public holidays) animals, a blood smear was prepared and stained with May-Grünwald-Giemsa stain. It was examined only when the results of Advia 120 determinations were abnormal.

Clinical Chemistry
Not evaluated in the present study.
Not required for carcinogenicity studies based on Guideline 870.4200 & OECD 451.

Urinalysis
Not evaluated in the present study.
Not required for carcinogenicity studies based on Guideline 870.4200 & OECD 451.

6. Bioanalytical examinations
During Weeks 52 and 78 of the study, a blood sampling (approximately 500 µL) was collected from the retro-orbital venous plexus of five suitable Isoflurane anesthetized animals from groups 2, 3 and 4 for each sex. Similarly, a blood sampling was also collected from 2 suitable male animals of Group 1 (controls). Animals were not overnight dietary fasted before blood sampling. The time point for blood collection (approximately 9:00 a.m.) was determined based on the results of a previous blood kinetic study conducted in Wistar rats.
Plasmas were prepared from blood collected into heparinized vials by centrifugation and stored in the dark at approximately -20 °C until shipment on dry ice for determination of test substance level and potentially its main metabolites at "Bioanalytical Investigations, Bayer CropScience AG, Development, Human Safety - Residue Analysis, [redacted], Germany" under the supervision on the Principal Investigator [redacted]. Shipping procedures followed the Standard Operating Procedures (SOPs) of the Testing Facility.

The samples were analyzed by High Performance Liquid Chromatography/Tandem Mass Spectrometry for concentrations of the active substance. The analytical part was performed in compliance with the actual Test Site relevant SOPs. The results of the investigation are presented in a separate bioanalytical phase report.

7. Sacrifice and pathology
On study days 370, 371 or 372 for the 12-month interim kill, and on study Days 552 to 566 for the carcinogenicity phase, all surviving animals dedicated to the interim sacrifice/chronic toxicity group and carcinogenicity phase group were sacrificed by exsanguination under deep anesthesia (Isoflurane). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were diet fasted overnight prior to sacrifice.

All animals, including animals at scheduled sacrifice, found dead, or sacrificed during the course of the study, were necropsied. The necropsy included the examination of all orifices, major organs, tissues and body cavities. All significant macroscopic findings were recorded.
Adrenal gland, brain, epididymides, heart, kidney, liver, ovary, spleen, testis, and uterus (including cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.
The following organs or tissues were sampled: adrenal gland, aorta, articular surface (femoro-tibial) bone (sternum), bone marrow (sternum), brain, epididymis, oesophagus, exorbital (lachrymal) gland, eye and optic nerve, gall bladder, harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina. Duplicate femoral bone marrow smear were prepared from sacrificed animals (except on weekends and public holidays), one of which was stained with May-Grunwald Giemsa, but not examined as no relevant changes were observed in haematology or bone marrow histology. The second smear was stored unstained for possible examination. Tissues samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative.
All the samples listed (except exorbital lachrymal gland, larynx/pharynx and nasal cavities) were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared from all organs and tissue samples, for histological examination for the carcinogenicity phase only.
Histotechnology was performed at "Propath UK limited, Willow Court, Netherwood Road, Hereford, HR2 6JU, England" under the supervision of the Principal Investigator, Nicola POWER, for the carcinogenicity phase.
No histopathology examination was done at the 52-weeks interim sacrifice. At terminal sacrifice, histopathological examinations were performed on all organs and tissues embedded including gross abnormalities in all animals from all groups including decedents. For all unscheduled sacrificed or dead animals on study, the cause of death was determined when it was possible.
Initial examinations were performed by the Study Pathologist. Following the initial examination, an external review pathologist ([redacted], U.K) undertook an independent « peer-review » of representative slides and diagnoses according to standardized operating procedures. The diagnoses presented in this report represent the consensus opinion of the two pathologists.

II. Results and discussion

A. Observations

1. Mortality

During the whole study period (at least 78 weeks), the mortality rate in animals allocated to the carcinogenicity phase of the study was comparable between the treated and control groups, with no statistically significant difference between dose groups.

During the study period, 1 female at 70 ppm died due to an accidental trauma and 1 female at 1500 ppm died during anesthesia for blood collection. These animals are included in the following table but were censored in the statistical analysis of the mortality rate.

Table 5.5.3-02: Mortality incidence over the whole study period for the carcinogenicity phase animals

BYI 02960 Dose Level (ppm)	Male	Female
0	11/50 (22%)	8/50 (16%)
70	5/50 (10%)	10/50 (20%)
300	9/50 (18%)	5/50 (10%)
1500	8/50 (16%)	7/50 (14%)

Percentage mortality in parentheses. Statistical analysis of the survival rate was performed at the end of the study on carcinogenicity phase animals.

2. Clinical signs of toxicity

There were no treatment-related clinical signs noted at the high (1500 ppm), mid (300 ppm) and low (70 ppm) dose levels in either sex.

A slightly higher incidence of skin lesions was noted in the high and mid dose male groups and of hair loss in the high dose male group, in comparison to controls. Since these findings were not dose-related and not statistically significant, they were considered to be incidental.

Table 5.5.3-03: Incidence of treatment-related clinical signs

Sex	Male				Female			
Dose level of BYI 02960 (ppm)	0	70	300	1500	0	70	300	1500
Group size	60	60	60	60	60	60	60	60
Skin lesions #	2 (3.3%) [2]	2 (3.3%) [2]	5 (8.3%) [5]	5 (8.3%) [5]	nc	nc	nc	nc
Hair loss #	25 (41.7%) [77]	25 (41.7%) [80]	24 (40.0%) [80]	34 (56.7%) [74]	nc	nc	nc	nc

nc : not concerned or no relevant change.
(%) : incidence.
[ ] : number of days of occurrence.
: Statistical analysis was conducted on this endpoint.

Other clinical signs recorded were those commonly recorded spontaneously in mice of this age and strain or were recorded in one or two animals only and were thus considered not to be related to BYI 02960 administration.

B. Body weight

Treatment-related changes in mean body weight parameters were noted consistently throughout the study at 1500 ppm and occasionally at 300 ppm in both sexes.

At 1500 ppm, mean body weight was progressively decreased in both sexes throughout the study by up to 7% in males ($p \leq 0.01$ on Weeks 38, 58, 66 and 70) and 8% in females ($p \leq 0.01$ on Week 66) compared to controls. Mean cumulative body weight gain was also significantly lower than controls throughout most study intervals in males (Weeks 1 to 2, 14 to 26 and 54 to 78) and from Week 14 onwards in females (Weeks 14 to 26, 26 to 54 and 54 to 78). Consequently, overall mean cumulative body weight gain between Days 1 to 540 (Weeks 1 to 78) was 19% lower than controls in males and 13% lower than controls in females.

At 300 ppm, mean body weight was similar to controls throughout the study, except on a few occasions where a slight decrease by up to 3% in males and 5% in females was observed, in comparison to controls. Mean cumulative body weight gain was also slightly lower than controls on a few occasions in both sexes throughout the study, resulting in an overall mean cumulative body weight gain between Days 1 to 540 (Weeks 1 to 78) 6% lower than controls in males and 10% lower than controls in females (not statistically significant). These changes on mean body weight parameters were considered not to be adverse in both sexes, in view of their low magnitude or occasional occurrences.

At 70 ppm, body weight parameters were unaffected by treatment in either sex.

Table 5.5.3-04: Mean body weights (BW) and cumulative body weight gains (BWG) (g)

Dose level of BYI 02960 (ppm)	0	70	300	1500
Male				
Initial BW (Day 1) (% C)	**20.6**	**20.6 (100)**	**20.6 (100)**	**20.6 (100)**
BW Week 2 (Day 8) (% C)	21.3	21.4 (100)	21.0 (99)	21.0 (98)
BW Week 14 (Day 92) (% C)	26.4	26.3 (100)	26.5 (101)	26.1 (99)
BW Week 26 (Day 176) (% C)	28.6	28.2 (98)	27.7 ** (97)	26.9 ** (94)
BW Week 54 (Day 372) (% C)	30.2	30.3 (100)	30.3 (100)	28.9 ** (95)
Final BW Week 78 (Day 540) (% C)	**31.2**	**30.9 (99)**	**30.7 (99)**	**29.4 ** (94)**
BWG Weeks 1-2 (Days 1 to 8) (% C)	**0.7**	**0.7 (106)**	**0.4 ** (57)**	**0.4 ** (60)**
BWG Weeks 1-14 (Days 1 to 92) (% C)	5.8	5.7 (98)	5.9 (102)	5.5 (95)
BWG Weeks 14-26 (Days 92 to 176) (% C)	2.2	1.9 (85)	1.2 ** (55)	0.8 ** (37)
BWG Weeks 26-54 (Days 176 to 372) (% C)	1.8	2.3 (125)	2.6 ** (142)	2.0 (112)
BWG Weeks 54-78 (Days 372 to 540) (% C)	0.9	0.6 (70)	0.3 * (34)	0.2 ** (24)
Overall BWG (Days 1 to 540) (% C)	**10.8**	**10.4(96)**	**10.2 (94)**	**8.7 ** (81)**
Female				
Initial BW (Day 1) (% C)	**17.2**	**17.0 (98)**	**16.9 (98)**	**16.8 (98)**
BW Week 2 (Day 8) (% C)	17.6	17.1 * (97)	17.1 * (97)	17.1 * (97)
BW Week 14 (Day 92) (% C)	22.0	22.3 (101)	22.2 (101)	22.0 (100)
BW Week 26 (Day 176) (% C)	23.7	24.1 (102)	23.3 (98)	23.1 * (97)
BW Week 54 (Day 372) (% C)	25.8	25.9 (100)	25.5 (99)	24.8 (96)
Final BW Week 78 (Day 540) (% C)	**28.5**	**28.4 (100)**	**27.1 (95)**	**26.6 ** (93)**
BWG Weeks 1-2 (Days 1 to 8) (% C)	**0.4**	**0.1 (39)**	**0.1 (36)**	**0.3 (75)**
BWG Weeks 1-14 (Days 1 to 92) (% C)	4.8	5.3 ** (112)	5.3 ** (112)	5.2 * (108)
BWG Weeks 14-26 (Days 92 to 176) (% C)	1.7	1.9 (107)	1.1 ** (63)	1.1 ** (63)
BWG Weeks 26-54 (Days 176 to 372) (% C)	2.1	1.9 (94)	2.2 (107)	1.7 (83)
BWG Weeks 54-78 (Days 372 to 540) (% C)	2.4	2.3 (99)	1.5 * (62)	1.6 * (67)
Overall BWG (Days 1 to 540) (% C)	**11.2**	**11.5 (103)**	**10.1 (90)**	**9.7 * (87)**

% C : % vs control (from raw data) * : $p \leq 0.05$ ** : $p \leq 0.01$ n c: not calculated.

C. Food consumption and compound intake

1. Food consumption

At 1500 ppm, mean food consumption was marginally decreased during the first two weeks of the study in males (- 4% on Week 1 and -3% on Week 2, not statistically significant) and on several occasions throughout the study in females (average decrease of 3%), compared to controls.

At 300 and 70 ppm, mean food consumption was unaffected by treatment in either sex.

Table 5.5.3-05:Group mean food consumption (g/animal/day)

Sex	Male				Female			
Dose level of BYI 02960 (ppm)	0	70	300	1500	0	70	300	1500
Week period 1 to13 (% control)	4.13 -	4.16 (101)	4.14 (100)	4.16 (101)	4.27 -	4.36 (102)	4.26 (100)	4.16 (97)
Week period 1 to 53 (% control)	4.10 -	4.13 (101)	4.11 (100)	4.11 (100)	4.24 -	4.31 (102)	4.24 (100)	4.14 (98)
Week period 1 to 77 (% control)	4.10 -	4.15 (101)	4.12 (101)	4.13 (101)	4.29 -	4.35 (102)	4.29 (100)	4.17 (97)

2. Achieved dosage

The mean achieved dietary intakes of BYI 02960 expressed in mg/kg body weight/day received by the animals during the study were as follows:

Table 5.5.3-06: Mean achieved dietary intake of BYI 02960 (mg/kg/day)

Sex	Male			Female		
Dose level of BYI 02960 (ppm)	70	300	1500	70	300	1500
Weeks 1 - 13	11.9	51	259	14.9	63	311
Weeks 1 - 52	10.5	45	232	12.9	56	276
Weeks 1 - 80	10.0	43	224	12.2	53	263

3. Food efficiency

Not evaluated in this study.

D. Ophthalmoscopic examination

Not evaluated in this study.

E. Blood analysis

1. Haematological findings

There were no relevant treatment-related changes noted at any dose level in either sex.

2. Clinical chemistry findings

Not evaluated in this study.

F. Urinalysis

Not evaluated in this study.

G. Bioanalytical examinations

Plasma samples were analyzed for BYI 02960 concentrations at Weeks 52 and 78. In the control group, all results were below the lower limit of quantitation. The values in the treated groups showed a dose-related increase in BYI 02960 concentration, with slightly higher levels in males than in females. There was no further accumulation after 78 weeks, since levels were similar to those measured after 52 weeks.

Table 5.5.3-07: Concentrations of BYI 02960 in plasma (mg/L) ± SD

Sex	Male				Female			
Dose-level of BYI 02960 (ppm)	0	70	300	1500	0	70	300	1500
Week 52	<LLOQ	1.29 ± 0.16	6.05 ± 0.92	27.4 ± 4.42	<LLOQ	0.94 ± 0.39	4.01 ± 1.08	24.8 ± 4.39
Week 78	<LLOQ	1.45 ± 0.13	6.58 ± 1.28	30.3 ± 1.68	<LLOQ	1.03 ± 0.22	3.90 ± 0.97	28.7 ± 3.56

<LLOQ : below the lower limit of quantitation of 25 µg/L

H. Sacrifice and pathology

1. Terminal body weight and Organ weights - 12-month interim sacrifice

A lower mean terminal body weight was observed at 1500 ppm in males only (- 6%, not statistically significant), when compared to controls. Mean terminal body weight was unaffected by treatment at 1500 ppm in females and at 300 and 70 ppm in either sex.

Treatment-related changes were noted in kidney and brain weights. Mean absolute kidney weight and mean kidney to brain weight ratio were statistically significantly lower than controls at 1500 ppm in males only. Mean brain to body weight ratio was slightly higher than controls at 1500 ppm in males only (+ 9%, $p \leq 0.05$), but this effect was related to the lower mean terminal body weight.

Table 5.5.3-08: Mean kidney weight ± SD at scheduled sacrifice (% change when compared to controls)

Mean kidney weight ± SD at scheduled sacrifice (% change when compared to controls)								
Sex	Male				Female			
Dose level of BYI 02960 (ppm)	0	70	300	1500	0	70	300	1500
Absolute kidney weight (g)	0.4867 ± 0.0337	0.4810 ± 0.0565 (- 1%)	0.4749 ± 0.0342 (- 2%)	0.4231 * ± 0.0471 (- 13%)	0.3565 ± 0.0429	0.3774 ± 0.0364 (+ 6%)	0.3523 ± 0.0525 (- 1%)	0.3491 ± 0.0246 (- 2%)
Kidney to body weight ratio (%)	1.7840 ± 0.1259	1.7809 ± 0.1213 (0%)	1.7956 ± 0.1255 (+ 1%)	1.6500 ± 0.1433 (- 8%)	1.6861 ± 0.0918	1.6436 ± 0.0924 (- 3%)	1.6479 ± 0.1337 (- 2%)	1.6386 ± 0.0935 (- 3%)
Kidney to brain weight ratio (%)	108.4771 ± 5.7003	107.5704 ± 13.7494 (- 1%)	106.8380 ± 8.7855 (- 2%)	92.3500 ** ± 9.0416 (- 15%)	76.6298 ± 6.3735	81.1747 ± 7.7034 (+ 6%)	76.1428 ± 9.2563 (- 1%)	74.6949 ± 5.3886 (- 3%)

* : $p \leq 0.05$ ** : $p \leq 0.01$

2. Organ weight - 18-month carcinogenicity phase

A lower mean terminal body weight was observed at 1500 ppm in males (- 4%, $p \leq 0.01$) and females (- 6%, not statistically significant), when compared to controls.

Treatment-related changes were noted in liver, kidney and brain weights. Mean absolute and relative liver weights were higher than controls at 1500 ppm in males. Mean liver to body weight ratio was also higher at 1500 ppm in females, but this effect was related to the lower mean body weight.

Table 5.5.3-09:Mean liver weight ± SD at scheduled sacrifice (% change when compared to controls)

Sex	Male				Female			
Dose-level of BYI 02960 (ppm)	0	70	300	1500	0	70	300	1500
Absolute liver weight (g)	1.128 ± 0.0784	1.128 ± 0.0846 (0%)	1.137 ± 0.0727 (+ 1%)	1.233 ** ± 0.0861 (+ 9%)	1.285 ± 0.1749	1.287 ± 0.1751 (0%)	1.220 ± 0.2035 (- 5%)	1.294 ± 0.1899 (+ 1%)
Liver to body weight ratio (%)	4.166 ± 0.2381	4.171 ± 0.2616 (0%)	4.172 ± 0.2388 (0%)	4.759 ** ± 0.2743 (+ 14%)	5.110 ± 0.3489	5.167 ± 0.4445 (+ 1%)	4.986 ± 0.6718 (- 2%)	5.509 ** ± 0.8036 (+ 8%)
Liver to brain weight ratio (%)	249.155 ± 16.3057	248.368 ± 19.3286 (0%)	251.190 ± 15.1611 (+ 1%)	270.814 ** ± 16.0455 (+ 9%)	272.958 ± 34.0733	270.002 ± 33.9804 (- 1%)	257.339 ± 44.1777 (- 6%)	273.926 ± 39.0896 (0%)

** : p ≤0.01

Mean absolute and relative kidney weights were statistically significantly lower than controls at 1500 ppm in males only.

Table 5.5.3-10: Mean kidney weight ± SD at scheduled sacrifice (% change when compared to controls)

Sex	Male				Female			
Dose-level of BYI 02960 (ppm)	0	70	300	1500	0	70	300	1500
Absolute kidney weight (g)	0.4959 ± 0.0482	0.5148 ± 0.0489 (+ 4%)	0.4932 ± 0.0505 (- 1%)	0.4290 ** ± 0.0384 (- 13%)	0.4424 ± 0.0497	0.4533 ± 0.0832 (+ 2%)	0.4381 ± 0.0455 (- 1%)	0.4187 ± 0.0420 (- 5%)
Kidney to body weight ratio (%)	1.8295 ± 0.1406	1.9004 ± 0.1321 (+ 4%)	1.8073 ± 0.1583 (- 1%)	1.6538 *** ± 0.0966 (- 10%)	1.7650 ± 0.1179	1.8233 ± 0.3017 (+ 3%)	1.7957 ± 0.1407 (+ 2%)	1.7800 ± 0.1246 (+ 1%)
Kidney to brain weight ratio (%)	109.5244 ± 10.1927	113.2376 ± 10.4639 (+ 3%)	108.8135 ± 9.7886 (- 1%)	94.2427 ** ± 7.5710 (- 14%)	93.9341 ± 8.6909	95.1333 ± 17.5359 (+ 1%)	92.2409 ± 8.8349 (- 2%)	88.5742 ± 7.4842 (- 6%)

** : p ≤0.01 *** : p ≤0.001

Mean brain to body weight ratio was slightly higher than controls at 1500 ppm in both sexes (+ 5% in males and + 7% in females, p ≤0.01), but this effect was related to the lower mean body weight.

The few other organ weight changes were considered to be incidental and not treatment-related.

3. Gross pathology - 12-month interim sacrifice

Unscheduled deaths

Five animals died before the end of the study.

One group 4 male was killed for humane reasons on study Day 295 with an abscess near the preputial gland.

In the group 3, one male was killed for humane reasons on study Day 329 with a distended urinary bladder, another male was found dead on study Day 174 with no observed macroscopic changes and one female was killed for humane reasons on study Day 233 with a cutaneous alopecia and an enlarged axillary lymph node.
One control male was killed for humane reasons on study Day 195 with a wound in the anogenital area, an enlarged spleen and a mass in the subcutis near the preputial gland.
All these changes were considered to be incidental and not treatment-related.

Terminal sacrifice
All gross pathology changes were considered as incidental and not treatment-related.

4. Gross pathology - 18-month carcinogenicity phase
Unscheduled deaths
Sixty-six animals died before the end of the study.
All macroscopic changes observed were considered to be incidental and not treatment-related.

Terminal sacrifice
At 1500 ppm in males, atrophic/small kidneys were noted in 5/42 animals, compared to no case in the controls ($p \leq 0.05$). This change was considered to be treatment-related and correlated with decreased or absence of corticoepithelial vacuolation.

Table 5.5.3-11: Incidence of macroscopic changes in the kidney at terminal sacrifice

Sex	Male				Female			
BYI 02960 Dose level (ppm)	0	70	300	1500	0	70	300	1500
Atrophic/small	0/38	0/45	0/41	5/42 *	0/42	0/39	0/45	0/42

** : $p \leq 0.01$ *** : $p \leq 0.001$

Other macroscopic changes observed were considered to be incidental and not treatment-related.

5. Microscopic pathology - 12-month interim sacrifice
Histopathology was not performed.

6. Microscopic pathology - 18-month carcinogenicity phase
Unscheduled deaths
For all animals, the cause of death was considered not to be treatment-related.

Terminal sacrifice
Treatment-related effects were noted in the liver and kidneys (results are shown for combined scheduled and unscheduled deaths).

Non-neoplastic findings
In the liver, a decreased incidence of diffuse hepatocellular macrovacuolation (mainly periportal) was noted at 1500 ppm in females. A higher incidence and severity of diffuse hepatocellular vacuolation (mainly centrilobular) was noted in males at 1500 ppm ($p \leq 0.01$) and 300 ppm (not statistically significant).

These changes were considered to be treatment-related but not adverse, as there were no associated degenerative changes.

Table 5.5.3-12: Incidence and severity of microscopic changes in the liver (all animals) - 18-month carcinogenicity phase

Sex	Male				Female			
Dose level of BYI 02960 (ppm)	0	70	300	1500	0	70	300	1500
Number of animals examined	50	50	50	50	50	50	50	50
Hepatocellular macrovacuolation : mainly periportal: diffuse								
Minimal	0	0	0	0	32	28	28	13
Slight	0	0	0	0	2	1	1	0
Total	**0**	**0**	**0**	**0**	**34**	**29**	**29**	**13****
Hepatocellular vacuolation : mainly centrilobular: diffuse								
Minimal	10	16	2	0	0	0	0	0
Slight	16	16	22	1	0	1	0	1
Moderate	2	2	12	31	0	1	0	3
Marked	0	0	0	5	1	0	0	0
Total	**28**	**34**	**36**	**41****	**1**	**2**	**0**	**4**

** : p≤ 0.01

In the kidney, at 1500 ppm in males, decreased incidence and severity of bilateral basophilic tubules, focal cortical mineralization and corticoepithelial vacuolation were noted. At 300 ppm in males, a decreased severity of corticoepithelial vacuolation was observed. These changes were considered to be treatment-related but not adverse (lower incidence and severity of a common finding observed in control animals).

Table 5.5.3-13: Incidence and severity of microscopic changes in the kidney (all animals) - 18-month carcinogenicity phase

Sex	Male				Female			
BYI 02960 Dose level (ppm)	0	70	300	1500	0	70	300	1500
Number of animals examined	50	50	50	50	50	50	50	50
Basophilic tubules : bilateral								
Minimal	23	23	18	3	3	2	3	1
Slight	4	2	3	0	0	0	0	0
Total	**27**	**25**	**21**	**3****	**3**	**2**	**3**	**1**
Cortical mineralization: focal								
Minimal	12	19	13	1	1	0	0	0
Moderate	0	0	1	0	0	0	0	0
Total	**12**	**19**	**14**	**1****	**1**	**0**	**0**	**0**
Corticoepithelial vacuolation								
Minimal	3	4	5	19	0	0	0	0
Slight	4	3	7	9	0	0	0	0
Moderate	29	26	31	4	0	0	0	0
Marked	13	14	4	0	0	0	0	0
Total	**49**	**45**	**47**	**32****	**0**	**0**	**0**	**0**

** : $p \leq 0.01$

Other histopathological changes were considered to be incidental and not treatment-related.

Neoplastic findings

No treatment-related tumor was observed.

III. Conclusions

In conclusion, dietary administration of BYI 02960 over an 18-month period to the C57BL/6J mouse at dose levels up to 1500 ppm (equivalent to 224 mg/kg/day in males and 263 mg/kg/day in females) did not induce carcinogenic effects.

A dose level of 300 ppm (equivalent to 43 mg/kg/day in males and 53 mg/kg/day in females) was considered to be a No Observed Adverse Effect Level (NOAEL) in both sexes over an 18-month period of dietary administration.

KIIA 5.5.4 - Mechanism of action and supporting data

As no treatment-related tumours were observed in both rats or mice, no mechanistic studies were undertaken.

KIIA 5.6 - Reproductive toxicity

All studies presented in this section were conducted between 2010-2012 and complied with OECD, EU, USEPA and Japanese MAFF testing guidelines and Good Laboratory Practice (GLP).

In the rangefinder one generation rat reproduction study, BYI 02960 was administered continuously in the feed to Wistar rats (10 animals/dose/sex) at nominal dietary concentrations of 0, 200, 700, and 2000 ppm. : Males exhibited a very slight decline in body weight gain over 15 weeks of treatment with the test substance at 2000 ppm. Females showed declines in absolute body weight and body weight gain as well as declines in food consumption throughout the premating period at 2000 ppm and decline in body weight gain at 700 ppm. Statistically significant body weight declines were also observed throughout gestation and lactation at 2000 ppm; at 700 ppm declines in body weight during lactation with significance observed by lactation Day 14 were observed. Females treated at 2000 ppm also exhibited test substance-related decreases in absolute and relative spleen weight. At 2000 and 700 ppm, declines in absolute male and female pup weight were observed beginning PND 14 and continuing to PND 21 with significance only observed for the females. Body weight gain for the males and females was also declined, relative to controls. No test substance-related findings were observed on reproductive parameters.

In the rat two-generation reproduction study, BYI 02960 was administered continuously in the diet to Wistar rats (30 animals/dose/sex) at nominal dietary concentrations of 0, 100, 500, and 1800 ppm. In the P-generation and F_1-generation, females from the 1800 ppm treated group exhibited declines in body weight during premating, gestation and lactation. In the P-generation males treated at 1800 ppm, increased absolute and relative liver weights were observed as well as increased absolute thyroid weights. Minimal centrilobular hypertrophy of the liver was observed in the males and correlated with the increased liver weights. Declines in body weight were also observed in the females treated at 500 ppm from the P-generation during the premating period and the females from the F_1-generation during premating, gestation and lactation periods.
F_1-offspring from the 1800 ppm parental group showed a significant decline in body weight at birth and during lactation. No decline in body weight was observed at birth for the F_2-offspring, but a significant decline was observed during lactation. In the F_1-offspring a significant delay in preputial separation and a slight nonstatistical delay in vaginal patency were observed in parallel with the decreased body weight. However, no effect on anogenital distance was observed in the F_2-generation pups. In both generations, variations in brain, thymus and spleen weights in males and/or females were observed and are considered to be due to the decreased body weights observed at this same dietary level and not a direct effect of the test substance. At 500 ppm a decline in body weight was observed in F_2-generation pups. Variations in brain, thymus and spleen weights in males and/or females were also observed in the F_2-ofsspring and are considered to be due to the decreased body weights.
A slight decrease in litter size was noted in the F_2-generation pups at 1800 ppm. The decline in litter size (9.2) is just outside of this laboratory's historical control range (9.8 - 11.8) and declines in total gain during gestation for the F_1-adults occurred concomitantly with this finding. Also, associated with the decrease in litter size was a decline in the total number of implantation sites in the F_1-adults, relative to controls. There was no test substance-related effects observed on the viability of the pups after delivery at any dietary level tested.

The parental systemic NOAEL was 500/100 ppm in males and females, respectively (32.3/7.8 mg BYI 02960/kg bw/day) based upon liver and thyroid effects in P-generation males and body weight effects in females. The reproductive NOAEL was 500 ppm (32.3/39.2 mg BYI 02960/kg bw/day in males and females, respectively) based upon decreased cycle number, litter size and number of implants in F_1 generation. The offspring NOAEL was 100 ppm (7.8 mg BYI 02960/kg bw/day) based upon body weight effects in F_2 pups.

In a rat developmental study, BYI 02960 was administered daily by gavage to groups of 25 pregnant Sprague-Dawley female rats per dose-group at 15, 50 and 150 mg/kg/day from gestation day (GD) 6 to 20. The control group received the vehicle alone, an aqueous solution of 0.5% methylcellulose 400. At 150 mg/kg/day, there was a mean maternal body weight loss of 5.7 g between GD 6-8, compared to a weight gain of 5.9 g in the concurrent controls. In addition, between GD 8-10, the mean body weight gain was reduced by 24% when compared to the control group. Mean food consumption was reduced by between 9 and 27% on all intervals between GD 6 and 12. At 50 mg/kg/day, the mean maternal body weight gain was reduced by 49% and mean food consumption was reduced by 8% between GD 6 - 8, when compared to the concurrent controls. At 150 mg/kg/day, the mean absolute liver weight was 13% higher than controls. At cesarean section, mean fetal body weights for combined sexes and females were marginally reduced compared to the controls (by 2 to 3%, not statistically significant). At the fetal skeletal examination, the incidences of two variations ("parietal (uni/bi): incomplete ossification" and "hyoid centrum: incomplete ossification") were higher than in the control group and were indicative of a slightly delayed fetal development. The NOEL for maternal toxicity was 15 mg/kg/day and the NOEL for developmental toxicity was 50 mg/kg/day.

In a complementary study, where groups of 23 sperm-positive female Sprague-Dawley rats were exposed to BYI 02960 by oral gavage from gestation day (GD) 6 to 20 at 20 and 30 mg/kg/day, no maternal toxicity was observed up to 30 mg/kg/day. Therefore, based on these two studies, it can be concluded that the NOEL for maternal toxicity was 30 mg/kg/day and the NOEL for developmental toxicity was 50 mg/kg/day.

In a rabbit developmental study, groups of 23 time-mated pregnant female New Zealand White rabbits were administered BYI 02960 by oral gavage from gestation day (GD) 6 to 28 at 7.5, 15 and 40 mg/kg/day. A dose level of 40 mg/kg/day BYI 02960 resulted in maternal toxicity as evidenced by body weight loss, significantly reduced body weight gain and food consumption between GD 6 and 10, and lower mean maternal corrected body weight change compared to control animals. Fetal development was unaffected by treatment at any dose level tested. A dose level of 15 mg/kg/day was considered to be a No Observed Adverse Effect Level (NOAEL) for maternal toxicity, while a dose level of 40 mg/kg/day was considered to be a NOAEL for developmental toxicity.

Results are summarized in tabular form below.

Type of study Doses	NOAEL (mg/kg/d)	LOAEL (mg/kg/d)	Adverse effects at LOAEL/ target organs	
Reproductive toxicity studies				
One-generation rat M-394208-01-2 0, 200, 700, 2000 ppm	50.1/17.5 ((M/F)	147.5/60 (M/F)	Parent	*Males:* Slight declines in BWG *Females:* Decreased BW and /or BWG (premating, gestation and lactation)
	147.5/168.9 (M/F)	>147.5/168.9 (M/F)	Repro-duction	No effects
	17.5	60. 9	Offspring	Decreased BW and BWG
Two-generation rat M-417665-01-2 0, 100, 500, 1800 ppm	32.3/7.8 (M/F) [500/100 ppm]	119.8/39.2 (M/F) [1800/500 ppm]	Parents	*Males:* Increased liver weights (P) Increased thyroid weights (P) Increased incidence of centrilobular hypertrophy (minimal - P) *Females:* Decreased BW (premating, gestation, and lactation; F_1) Decreased BWG (premating; P and F_1) Decreased terminal body weights (P & F_1)
	32.3/39.2 (M/F) [500/500 ppm]	119.8/140.2 (M/F) [1800/1800 ppm]	Repro-duction	Decreased cycle number (F_1), litter size (F_1), and number of implants (F_1)
	7.8 (M/F) [100 ppm]	39.8 (M/F) [500 ppm]	Offspring	Decreased BW and BWG (F_2); with Secondary to BW decreases: organ weight changes in brain, thymus, and spleen
Developmental toxicity studies				
Developmental toxicity Rat M-363938-01-2 0, 15, 50, 150 mg/kg/d	15 (Maternal)	50	Dams	Decreased mean BWG and food consumption (FC)
	50 (Develop.)	150	Fetuses	Decreased fetal BW; Reduced ossification of a few skull bones
Complementary rat toxicity M-425810-01-2 0, 20, 30 mg/kg/d	30	>30	Dams	No maternal toxicity
Developmental toxicity rabbit, M-423559-01-1 0, 7.5, 15, 40 mg/kg/d	15 (Maternal)	40	Dams	Decreased BW, BWG, corrected BWG, and FC (GD6-10)
	40 (Develop.)	>40	Fetuses	No treatment-related effects

KIIA 5.6.1 - Two generation reproductive toxicity in the rat

Report:	**KIIA 5.6.1 /01; [redacted];2011**
Title:	Technical Grade BYI 02960: A Dose Range-Finding Reproductive Toxicity Study in the Wistar Rat
Report No.: Document No.:	09-P72-RB M-394208-01-2
Guidelines:	None - pilot study
Deviations:	No
GLP	Yes

Executive Summary

The principal objective of this reproduction toxicity study was to determine appropriate dietary levels for a definitive (guideline) two-generation reproductive toxicity study with BYI 02960. In this study, BYI 02960 was administered continuously in the feed to the Wistar rat (10 animals/dose/sex) at nominal dietary concentrations of 0, 200, 700, and 2000 ppm. All test diets (including control) were available for *ad libitum* consumption; the homogeneity and stability of BYI 02960 as a dietary admixture was confirmed. Body weight and food consumption determinations and detailed clinical examinations of each animal were conducted weekly throughout the study, as well as, an evaluation of multiple reproductive parameters. All animals placed on study were subject to a postmortem examination, which included documenting and saving all gross lesions, weighing designated organs and, collecting representative tissue specimens. Micropathology was not performed on any tissue collected in this study.

Effects attributed to exposure to BYI 02960 were as follows:

<u>At 2000 ppm</u>

P-generation Adults: Males exhibited a very slight decline in body weight gain over 15 weeks of treatment with the test substance. Females showed declines in absolute body weight and body weight gain as well as declines in food consumption on a gram/animal/day basis throughout the premating period. Significant body weight declines were observed throughout gestation and lactation. Females exhibited test substance-related decreases in absolute and relative spleen weight.

F_1-Offspring: Declines in absolute male and female pup weight were observed beginning PND 14 and continuing to PND 21 with significance only observed for the females. Body weight gain for the males and females was also declined, relative to controls.

Reproductive Performance (F_0 and F_1): No test substance-related findings were observed.

<u>At 700 ppm</u>

P-generation Adults: Females exhibited declines in body weight gain throughout the premating period. A slight nonstatistical decline in body weight was also noted for the females during gestation as well as declines in body weight during lactation with significance observed by lactation Day 14.

F_1-Offspring: Declines in absolute male and female pup weight were observed beginning PND 14 and continuing to PND 21 with significance only observed for the females. Body weight gain for the males and females was also declined, relative to controls.

Reproductive Performance (P and F_1): No test substance-related findings were observed.

At 200 ppm
P-generation Adults: No test substance-related findings were observed.

F_1-Offspring: No test substance-related findings were observed.

Reproductive Performance (P and F_1): No test substance-related findings were observed.

Conclusions
The parental systemic LOAEL is 2000/700 ppm (147.5/60.0 mg BYI 02960/kg bw/day in males/females, respectively) based on slight declines in body weight gain in males and decreased body weight and/or body weight gain in females during premating, gestation, and lactation. The parental systemic NOAEL was 700/200 ppm (50.1/17.5 mg BYI 02960/kg bw/day in males/females, respectively).

The reproductive NOAEL was the high dietary level of 2000 ppm in both sexes (147.5/168.9 mg BYI 02960/kg bw/day in males/females, respectively) due to the absence of treatment-related findings on reproductive parameters (LOAEL > 2000 ppm; >147.5/168.9 mg BYI 02960/kg bw/day in males/females, respectively).

The offspring LOAEL is 700 ppm (60.9 mg BYI 02960/kg bw/day) based on decreases in pup body weight and body weight gain in both sexes. The offspring NOAEL is 200 ppm (17.5 mg BYI 02960/kg bw/day).

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Beige powder
Lot/Batch: 2009-000239
Purity: 96.2% (Date of analysis: 16 January 2009)
CAS: 951659-40-8
Stability: Stable in the diet over a concentration range of 20-2500 ppm when stored for 7 days at room temperature and following freezer storage for a minimum of 28 days (Moore, 2010).

2. Vehicle control: Acetone
Manufacturer: Fisher Scientific Optima
Lot Numbers (Expiration): 086527 (9 December 2011)
090732 (6 March 2011)
092136 (28 April 2012)

3. Test animals:
Species: Rat
Strain: Wistar Han CRL: WI (HAN)
Age at study initiation: (P) 8-9 weeks
Weight at randomization: Males: 156.1 - 190.5 g, Females: 122.2 - 145.2 g
Source: [redacted] US
Acclimation period: Minimum of six days
Diet: Purina Mills Certified Rodent Diet 5002 in "meal" form.
The "Certification Profile" for Purina Mills Certified Rodent Diet 5002M provided by the vendor is kept on file at [redacted].
No contaminants were present in the food in sufficient quantities to affect the conduct or results of the study.
Available *ad libitum*.
Water: Tap water (Kansas City, MO), *ad libitum*.
Housing: Animals were housed individually (except during the mating phase) in suspended stainless steel cages with a deotized cage board in the bedding trays. During gestation and lactation, individual dams (and their litter) were housed in polycarbonate cages with corn cob bedding.
Environmental condition: Temperature: 18 to 26 °C
Humidity: 30 to 70%
Air changes: Minimum daily average of 11.85 air changes per hour
Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates
30 March 2009 to 3 August 2009 at [redacted], USA.

2. Animal assignment and treatment
Following a minimum of six days of quarantine/acclimation, animals were examined by a veterinarian and released for study use. The animals were assigned to either a control or one of three chemically-treated groups using a weight stratification based computer program [DATATOX (Version rC.10), Instem Computer Systems, P/C, Stone, Staffordshire, England]. Only those animals falling within +/- 20% of the mean for all animals of each gender were placed on study. Once animals were assigned to their dose groups, each rat on study had a microchip (Biomedical Data Systems, Inc., Seaford, DE) subcutaneously implanted on its dorsal surface in the region between its scapulae. At a minimum, the chip was encoded with a unique number, specifying the animal's sex, dose group, cage number, and study affiliation. In addition, a laminated cage card, essentially duplicating the information present on

the chip, was attached to the outside of each animal's cage. Pups born alive were identified by tattoo and pups found dead were identified with a marking pen.

Study schedule

Forty female and forty male rats were assigned to one of four treatment groups (10 rats/sex/group; Table 5.6.1-1): nominal doses of 0, 200, 700, and 2000 ppm BYI 02960 mixed in the diet. Animals were exposed to the treated feed throughout the entire in-life phase of the study. In-life phases include: Premating: 10 weeks; Mating: 7 days; Gestation: approximately 22 days; and Lactation: weaning on Day 21.

Table 5.6.1-01: Animal Assignment

Test Group	Dose in Diet [a] (ppm)	Animals/group	
		P Male	P Female
Control	0	10	10
Low (LDT)	200	10	10
Mid (MDT)	700	10	10
High (HDT)	2000	10	10

[a] : The test material will be administered from beginning of the study until sacrifice.
LDT : Low dose tested MDT = Mid-dose tested HDT = High dose tested

Mating procedure

Males and females were exposed to the test substance for ten weeks prior to mating. Mating was accomplished by co-housing one female with one male for up to 7 consecutive days. During the mating phase, vaginal smears were taken each morning and examined for the presence of sperm and/or internal vaginal plug. Females found to be inseminated were placed in a polycarbonate nesting cage. The day on which insemination was observed in the vaginal smear was designated Day 0 of gestation for that female. In order to evaluate those females that might have been inseminated without exhibiting sperm in the vaginal smear or an internal vaginal plug, all remaining females were placed in polycarbonate nesting cages, following the 7-day mating period.

3. Dose selection rationale

Dietary levels were selected based on results from a 90-day rat study conducted with the test substance at dietary levels of 0, 100, 500, and 2500 ppm BYI 02960/kg body weight/day (M-329048-03-2). In that study, the following findings were considered to be attributed to the test substance. At 2500 ppm, decreased body weight, body weight gain, and food consumption were observed in both males and females. Enlarged livers with minimum to slight centrilobular hypertrophy were observed in both sexes upon microscopic examination. Various changes in the clinical chemistry profile were also observed in both sexes at 2500 ppm, including increased cholesterol and triglycerides, and decreased glucose and total bilirubin. Based on these results, doses selected for this dose range-finding reproduction study were 0, 200, 700, and 2000 ppm.

4. Dosage preparation and analysis

The test substance was dissolved in acetone and then mixed with the feed. Treated diet was mixed at room temperature; aliquots of the chemical were taken from the original test batch and transferred to the

mixing area. The control test diet was prepared in the same manner as the chemically-treated test diet, excluding only the test substance. A sample of each batch of feed mixed was taken and retained in the freezer until the study was complete and the analytical data deemed satisfactory. Replacement admixtures for each treatment group were prepared weekly (or at greater intervals depending on freezer stability) and stored under freezer conditions until presented to the animals the following week (or weeks).

The concentration of the test substance in the feed for the females only was adjusted during the lactation period (Days 0-21) by 50%. Samples from the first batch of adjusted feed for each dietary level were analyzed to measure the concentration. During the lactation phase, a substantial increase in food consumption is observed in all dams, which results in greatly increased intake of test substance (normal occurrence). A decrease in the dietary concentration of the test substance offsets this increased food consumption, thereby maintaining an approximately constant test substance intake (mg/kg body weight/day) throughout the study.

The mean daily intake of the test substance (mg BYI 02960/kg bw/day) throughout this one-generation reproduction study at nominal concentrations of 0, 200, 700, or 2000 ppm is summarized in Table 5.6.12.

Table 5.6.1-02: Mean Daily Intake of the Test Substance

Phase of Study	200 ppm in mg/kg/day[a]	700 ppm in mg/kg/day[a]	2000 ppm in mg/kg/day[a]
Premating (P-gen) - Male	14.5	50.1	147.5
Premating (P-gen) - Female	17.5	60.0	168.9
Gestation (P-gen) - Female	15.8	48.8	164.4
Lactation (P-gen) - Female	17.5	60.9	182.3

[a] : Individual values were based on the means for each particular phase

The concentration of BYI 02960 in the various test diets was analytically verified for batches intended for weeks 1 and 2, 5 and 6, 9 and 10, 12, 14, and 16 and 17 (Bayer CropScience LP, Environmental Research, [redacted]). Test diets intended for the first week of lactation were also analyzed.

Homogeneity Analysis

The mean concentrations of BYI 02960 in the feed, sampled from three distinct layers in the mixing bowl and containing a nominal concentration of either 20- or 2500-ppm, were determined to be 19.0 ppm (range 18.4 - 19.4 ppm; % RSD = 1.83) and 2445 ppm (range 2361 - 2505 ppm; % RSD = 1.6), respectively. Based on a %RSD ≤ to 10%, BYI 02960 was judged to be homogeneously distributed in the feed over a concentration range of 20 - 2500 ppm ([redacted], 2010).

Stability Analysis

Following 7 days of room temperature storage, the analytically-determined concentration of the AI of the test substance in the 20 or 2500 ppm admixture was determined to be 19.8 ppm (19.2 ppm on Day 0) and 2632 ppm (2445 ppm on Day 0), respectively. Following 28 days of freezer storage, the analytically-determined concentration of the AI of the test substance in the 20- and 2500-ppm admixtures was determined to be 19.1 ppm (19.0 on Day 0) and 2531 ppm (2445 on Day 0), respectively. BYI 02960 mixed in rodent ration was judged to be stable at room temperature for at least 7 days and following freezer storage for a minimum of 28 days, over a concentration range of 20 - 2500 ppm (Moore, 2010).

Concentration Analysis

Mean analytical concentrations for each dose group were 199, 684, and 1975 ppm, ranging from 98 - 100% of the corresponding nominal concentrations of 200, 700, and 2000 ppm, respectively. During lactation, the concentration of the test substance in the feed for the females was adjusted by 50%. Mean analytical concentrations for each dose group during lactation were 102, 355, and 998 ppm, ranging from 100-102% of the corresponding nominal concentrations of 100, 350, and 1000 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 114% and ranged from 110 - 125% for rodent ration spiked with 100, 200, 350, 700, 1000, or 2000 ppm of BYI 02960 (Quincy, 2010).

5. Statistics and calculation of reproductive and offspring indices

Statistical analyses

The data were analyzed using applications provided by DATATOX, SAS (Version 6.09 Enhanced, SAS Institute Inc., Cary, North Carolina), or TASC (Toxicology Analysis Systems Customized, 1993, Scientific Computer Consultants, New Jersey). Parametric data (including body weight gain and food consumption) were analyzed using a univariate Analysis of Variance (ANOVA), and if significant differences were observed, a Dunnett's Test was performed. Nonparametric data (*e.g.*, number of estrous cycles, litter size, and number of implantation sites) were first analyzed by the Kruskal-Wallis test and then subjected to Dunn's Test if significant differences were identified. Nonparametric dichotomous data (*e.g.*, fertility and gestation indices) were initially analyzed by the Chi-Square Test and if significance was observed between groups then by the Fisher's Exact Test with the Bonferroni adjustment. To the extent possible, the frequency of gross lesions were first examined visually, then in the event of questionable distribution, by statistical analyses using the Chi-Square and Fisher's exact tests. Differences between the control and test-compound-treated groups were considered statistically significant when $p \leq 0.05$ or $p \leq 0.01$.

Reproductive indices

The following reproductive indices were calculated from breeding and parturition records of animals in the study:

$$\text{Mating Index (\%)} = \frac{\text{\# inseminated females}^{a}}{\text{\# of females co-housed}} \times 100$$

$$\text{Fertility Index (\%)} = \frac{\text{\# of pregnant females}^{b}}{\text{\# of inseminated females}} \times 100$$

$$\text{Gestation Index (\%)} = \frac{\text{\# of females with live pups}}{\text{\# of pregnant females}} \times 100$$

[a] Includes pregnant females not observed sperm positive or with an internal vaginal plug.

[b] Includes females that did not deliver, but had implantation sites.

Offspring viability indices

The following viability indices were calculated from lactation records of litters in the study:

$$\text{Birth Index (\%)} = \frac{\text{total \# of pups born/litter}}{\text{total \# of implantation sites/litter}} \times 100$$

$$\text{Live birth Index (\%)} = \frac{\text{\# of live pups born/litter}}{\text{total \# of pups/litter}} \times 100$$

$$\text{Viability Index (\%)} = \frac{\text{\# of live pups/litter on day 4 (pre-culling)}}{\text{\# of live pups born/litter}} \times 100$$

$$\text{Lactation Index (\%)} = \frac{\text{\# of live pups/litter on day 21}}{\text{\# of live pups/litter on day 4 (post-culling)}} \times 100$$

Gestation Length = Number of whole days from day in which insemination is observed in the vaginal smear (Day 0 of gestation) to Lactation Day 0 (delivery of pups and entry in computer system)

C. Methods

1. Parental animals

Mortality and clinical observations

Mortality checks (cage-side observations) were performed twice daily (AM and PM), during the workweek and once daily on weekends and holidays. Cage-side observations characterized mortality, moribundity, behavioral changes, signs of difficult or prolonged delivery, and overt toxicity by viewing the animal in the cage. In the event a possible clinical sign was observed during the cage-side evaluation, the animal may have been removed from the cage and a detailed assessment conducted. A

detailed evaluation of clinical signs included both observing the animal in the cage and removing the animal to perform a physical examination. This was conducted at least once per week throughout the entire in-life phase of the study.

Body weights and food consumption

Body weight and food consumption was measured and fresh feed provided once per week for both males and females during the 10-week premating period. During the mating period and until sacrifice, body weight for the males and unmated females was measured once per week. Also during the mating period, fresh feed was provided for both males and unmated females once each week without measuring food consumption. During gestation, dam body weight was measured on Days 0, 6, 13, and 20 and fresh feed was provided and food consumption measured once each week. During lactation, dam body weight was measured on Days 0, 4, 7, 14, and 21. Fresh feed was provided and food consumption measured once per week, with the exception of week one when food consumption was measured twice (Days 0-4 and 4-7).

Estrous cyclicity

Estrous cycle staging was not performed in this dose range-finding study.

Sperm parameters

Sperm analysis was not performed in this dose range-finding study.

2. Litter observations

The following litter observations (X) were made (see Table 5.6.1-03).

Table 5.6.1-03: F_1 Litter Observations

Observation	Time of Observation (lactation day)					
Number of live pups	Day 0	Day 4	Day 7	Day 14	Day 21	Days (0 - 21)
Number of live pups						X
Pup weight	X	X	X	X	X	
External alterations						X
Number of dead pups						X
Sex of each pup (M/F)	X					

The size of each litter was adjusted on lactation Day 4 to yield, as closely as possible, four males and four females per litter. If the number of male or female pups was less than four, a partial adjustment was made (*e.g.*, three females and five males). No adjustment was made for litters of fewer than eight pups. Adjustments were made by random selection of the pups using software provided by SAS. Culled pups were sacrificed by decapitation. Grossly abnormal pups underwent a gross internal and external examination, and all culled pups were discarded. The pups not culled on lactation Day 4 were maintained with the dam until weaning on lactation Day 21. At weaning, pups were sacrificed by carbon dioxide asphyxiation and examined macroscopically for any structural abnormalities or pathological changes, particularly as they may relate to the organs of the reproductive system. Histopathology was not performed on any collected tissues.

Pup viability and clinical signs

Mortality checks (cage-side observations/pup counts) were performed once daily (AM), during the workweek and on weekends and holidays. Cage-side observations characterized mortality, moribundity, behavioral changes and overt toxicity by viewing the pups in the cage. In the event a possible clinical sign was observed during the cage-side evaluation, the pups may have been removed from the cage and a detailed assessment conducted. A detailed evaluation of clinical signs included both observing the pups in the cage and removing the animals to perform a physical examination and were conducted daily (Day 0-21), unless animals were retained for further study purposes.

3. Postmortem observations:

Parental animals

All surviving parental males were sacrificed as soon as possible after the last litters were born. Maternal animals were sacrificed following the weaning of their respective litters (lactation Day 21). The animals were subjected to postmortem examinations as follows:

Males were euthanized by carbon dioxide asphyxiation and a gross external examination was performed. Terminal body weights were measured and the recording of all gross pathologic alterations, weighing designated organs, and saving all gross lesions was conducted on all males.
Each dam was euthanized by carbon dioxide asphyxiation and a gross external examination was performed. Terminal body weights were measured and the recording of all gross pathologic alterations, weighing designated organs, and saving all gross lesions was conducted on all females. The uterus was excised and the implantation sites, if present, were counted.

Females that were sperm positive and/or had an internal vaginal plug but did not deliver were sacrificed after gestation Day 24. Females never observed as being inseminated and/or with an internal vaginal plug but did not deliver at least 24 days after the completion of the mating phase, were sacrificed and necropsied. A gross necropsy was performed on these animals as described above. In addition, patency of the cervical/uterine os in these females was examined via flushing of the uterine horns with 10% buffered formalin.

As summarized in Table 5.6.1-04, the following tissues were collected (X), or collected and weighed (XX).

Table 5.6.1-04: Tissues collected and/or weighed from parental animals

XX	Brain	XX	Epididymis	X	Lung
XX	Pituitary	X	Coagulating Gland	X	Physical Identifier
XX	Liver	XX	Ovary	X	Vagina
XX	Kidney	X	Oviduct	X	Cervix
XX	Spleen	XX	Prostate	X	Gross Lesions
XX	Thyroid	XX	Seminal Vesicle (with coagulating gland)		
XX	Thymus	XX	Testis		
XX	Adrenal	XX	Uterus (with oviduct and cervix)		

Histopathology was not performed on any tissue.

Animals found moribund while on study were sacrificed and a gross necropsy performed. Animals found dead were necropsied as soon as possible. Necropsy examinations included those parameters previously described. Pups found dead, stillborn or terminated in a moribund condition underwent a gross necropsy for possible defects and/or to determine the cause of death.

Offspring

The F_1-offspring were sacrificed at 21 days of age. These animals were subjected to postmortem examinations (macroscopic). The following tissues from 21-day weanlings were collected and weighed: brain, thymus, spleen, and uterus. Any gross lesion was documented and collected.
Pups found dead or terminated in a moribund condition underwent a gross necropsy for possible defects and/or cause of death.

II. Results and discussion

A. Parental animals

1. Mortality and clinical signs

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

2. Body weight and food consumption

Male (premating)

A very slight decline in body weight gain (- 9.3% when compared to controls) was observed in the 2000 ppm dose group. There was no test substance-related effects observed on absolute body weight at any dietary level tested. No test substance-related findings were observed on food consumption during the 10-week premating period at any dietary level tested.

Female (premating)

Declines in absolute body weight were observed in the 2000 ppm dose group beginning week two and continuing throughout the premating period (mean decline week 2-10 of 9.4%). Declines in body weight gain throughout the premating period were noted in the 700 and 2000 ppm dose groups (- 12.3% and - 33.4% compared to controls, respectively). Food consumption on a gram/animal/day

basis was decreased 8.8% in the 2000 ppm dose group, relative to controls, during the 10-week premating period. There were no test substance-related findings noted at the 200 ppm dietary level.

Reported body weight and selected food consumption results for premating are summarized in Table 5.6.1-05.

Table 5.6.1-05: Mean (S.E.) body weight and food consumption[a]

Observations/study week	Dose Group			
	Control 0 ppm	LDT 200 ppm	MDT 700 ppm	HDT 2000 ppm
P Generation Male				
Mean body weight (g) - Week 15 S.E.	432.9 13.42	463.2 14.30	454.6 11.51	432.6 14.36
Mean weight gain (g) Weeks 1-15	232.2	242.3	235.3	210.7
Mean food consumption (g/animal/day) Weeks 1-10	23.9	24.3	23.8	35.6
Mean food consumption (g/kg/day) Weeks 1-10	72.6	73.2	73.5	75.4
P Generation Female - Pre-mating				
Mean body weight (g) - Week 10 S.E.	231.0 3.87	230.3 5.22	221.7 6.00	207.0** 3.27
Mean weight gain (g) Weeks 1-10	79.0	79.5	69.3	52.6
Mean food consumption (g/animal/day) Weeks 1-10	17.1	17.2	16.8	15.6
Mean food consumption (g/kg/day) Weeks 1-10	86.8	88.4	88.4	85.7

[a] : Food consumption data represents grand means.
** : Statistically different from control, $p \leq 0.01$

Gestation

Significant body weight declines were observed in the 2000 ppm dose group throughout gestation (mean decline GD 0-20 of 11.3%). A slight nonstatistical decline in body weight was also noted in the 700 ppm dose group (mean decline GD 0-20 of 4.5%). No effects considered to be test substance-related were observed on body weight gain or food consumption during gestation at any dietary level tested.

Reported body weight and selected food consumption results during gestation are summarized in Table 5.6.1-06.

Table 5.6.1-06: Mean (S.E.) body weight and food consumption during gestation[a]

P Generation Female - Gestation				
Observations/study week	**Dose Group**			
	Control 0 ppm	**LDT 200 ppm**	**MDT 700 ppm**	**HDT 2000 ppm**
Mean body weight (g) - Day 0 S.E.	233.5 4.33	231.3 6.22	225.5 4.69	206.4** 4.47
Mean body weight (g) - Day 6 S.E.	249.4 5.26	251.8 6.06	241.2 4.09	225.0** 3.85
Mean body weight (g) - Day 13 S.E.	274.7 5.15	274.8 7.39	261.4 4.26	241.6** 4.38
Mean body weight (g) - Day 20 S.E.	334.8 6.45	337.8 10.78	315.5 6.10	295.9** 4.42
Mean weight gain (g) - Days 0 - 20 S.E.	101.4 5.30	106.5 6.14	90.0 3.98	89.5 3.15
Mean food consumption (g/animal/day) Days 0 - 20	18.6	20.1	17.4	18.6
Mean food consumption (g/kg/day) Days 0 - 20	73.8	80.1	71.8	83.1

[a] : Food consumption data represents grand means
** : Statistically different from control, $p < 0.01$

Lactation

Significant declines in body weight were observed in the 2000 ppm dose group throughout lactation (mean decline LD 0-21 of 10.2%). Declines in body weight were also observed in the 700 ppm dose group with significance by lactation Day 14 (mean decline LD 14-21 of 10.6%). No effects on body weight were noted in the 200 ppm dietary level. No test-substance-related effects were observed on food consumption during lactation at any dietary level tested.

Reported body weight and selected food consumption results for lactation are summarized in Table 5.6.1-07.

Table 5.6.1-07: Mean (S.E.) body weight and food consumption during lactation[a]

P Generation Female - Lactation				
Observations/study week	**Dose Group**			
	Control 0 ppm	**LDT 200 ppm**	**MDT 700 ppm**	**HDT 2000 ppm**
Mean body weight (g) - Day 0 S.E.	255.2 5.94	253.2 7.97	242.1 2.99	223.9** 5.85
Mean body weight (g) - Day 4 S.E.	266.1 4.96	270.0 7.13	253.6 4.16	241.6* 5.23
Mean body weight (g) - Day 7 S.E.	274.9 4.22	275.9 5.84	266.5 3.43	252.3* 5.39
Mean body weight (g) - Day 14 S.E.	296.6 4.78	296.4 6.25	276.8* 3.77	262.5** 6.28
Mean body weight (g) - Day 21 S.E.	290.1 4.80	287.1 10.04	272.8* 2.87	261.8** 3.95
Mean food consumption (g/animal/day) Days 0 - 21	49.3	47.3	45.0	45.3
Mean food consumption (g/kg/day) Days 0 - 21	179.3	171.3	171.5	182.7

[a]: Food consumption data represents grand means
*: Statistically different from control, $p \leq 0.05$
**: Statistically different from control, $p \leq 0.01$

3. Test substance intake

Based on food consumption, body weight and dietary analyses results, the doses expressed as mean daily mg test substance/kg body weight during the pre-mating period (10-weeks for males and females) are presented in Table 5.6.1-08.

Calculation for test substance intake is:
[Mean analytical concentration (ppm) specific for premating / 1000] X mean weekly food consumption (g/kg/body weight/day) during premating.

Table 5.6.1-08: Mean test substance intake during premating (mg/kg body weight/day)

Male			Female		
LDT 200 ppm	**MDT 700 ppm**	**HDT 2000 ppm**	**LDT 200 ppm**	**MDT 700 ppm**	**HDT 2000 ppm**
14.5	50.1	147.5	17.5	60.0	168.9

4. Reproductive Function

Estrous cycle length and periodicity

Estrous cycle staging was not performed in this dose range-finding study.

Sperm measures

Sperm analysis was not performed in this dose range-finding study.

5. Reproductive performance

There was no test substance-related effect on any reproductive parameter (*e.g.*, mating, fertility, or gestation indices, days to insemination, gestation length, or the median number of implants) at any dietary level tested. Table 5.6.1-09 below summarizes reproductive performance in this study.

Table 5.6.1-09: Reproductive performance in P-Generation animals

Observation	Dose Group (ppm)			
	Control 0 ppm	LDT 200 ppm	MDT 700 ppm	HDT 2000 ppm
P Generation - F_1 Offspring				
Number Cohoused	10	10	10	10
Number Mated	10	9	9	9
Number of Animals Delivered	8	8	9	9
Number of Animals with Implants	9	8	9	9
Mating Index	100.0	90.0	90.0	90.0
Fertility Index	90.0	88.9	100.0	100.0
Gestation Index	88.9	100.0	100.0	100.0
Mean Number Days to Insemination (S.E.)	2.8 (0.32)	1.9 (0.26)	2.3 (0.33)	2.2 (0.28)
Mean Gestation Length (days) (S.E.)	21.9 (0.23)	22.3 (0.16)	22.0 (0.24)	22.2 (0.28)
Median Gestation Length (days)	22.0	22.0	22.0	22.0

6. Parental postmortem results

Terminal body weight and organ weights

Male

Terminal body weight effects were not observed at any dietary level tested. Organ weight effects considered to be test substance-related were not observed at any dietary level tested.

Equivocal increases in liver weights were observed in both the 700 and 2000 ppm dose groups.

Female

Terminal body weight effects were not observed at any dietary level tested. Test substance-related decreases in absolute and relative spleen weight were observed in the 2000 ppm dose level. Organ weight effects considered to be test substance-related were not observed at any other dietary level tested.

Pathology

Macroscopic examination

No test substance-related gross necropsy findings were observed at any dietary level tested.

Microscopic examination

Histopathology was not performed for the parental tissues in this study.

B. Offspring

1. Viability and clinical signs

There were no test substance-related effects observed on the viability of the pups after delivery at any dietary level tested. No test substance-related clinical observations were observed at any dietary level tested.

Mean litter size and viability (survival) results from pups during lactation are summarized in Table 5.6.1-10.

Table 5.6.1-10: Litter parameters for Pups during lactation

Observation	Dose Group (ppm)			
	Control 0 ppm	LDT 200 ppm	MDT 700 ppm	HDT 2000 ppm
P Generation				
Total number of implantation sites	96	99	101	94
Total number of pups born	91	97	100	92
Number stillborn	0	4	0	0
Sex Ratio Day 0 (% male)	55.3	45.5	48.0	54.2
Mean litter size Day 0	11.4	12.1	11.1	10.2
Birth index	87.0	98.1	99.0	98.0
Live birth index	100.0	96.2	100.0	100.0
Viability index	100.0	98.2	99.1	100.0
Lactation index	100.0	100.0	100.0	100.0

2. Pup body weight (combined male and female)

F_1-Pups: There was no test substance-related effect observed on birth weight at any dietary level tested. Declines in absolute pup weight were observed beginning PND 14 and continuing to PND 21 in the 700 and 2000 ppm dietary groups with significance only observed for the females. Body weight gain for the males and females was declined in both the 700 and 2000 ppm dose group 9.7% and 10.8%, respectively.

Selected mean pup body weight data are presented in Tables 5.6.1-11, 5.6.1-12, and 5.6.1-13 for combined sexes and male and female pups individually, respectively.

Table 5.6.1-11: Mean (S.E.) combined male and female pup weights

F_1 Generation				
Lactation Day	**Control 0 ppm**	**LDT 200 ppm**	**MDT 700 ppm**	**HDT 2000 ppm**
0 S.E	6.1 0.18	6.0 0.18	5.7 0.11	6.0 0.23
4[a] S.E.	10.2 0.48	10.0 0.39	9.4 0.22	10.0 0.43
4[b] S.E.	10.1 0.51	10.0 0.39	9.4 0.22	10.0 0.41
7 S.E.	16.2 0.71	16.2 0.51	15.0 0.25	15.3 0.45
14 S.E.	33.2 0.86	32.1 0.83	29.9* 0.57	29.9* 0.71
21 S.E.	50.6 1.42	48.4 1.05	45.9 1.11	45.7* 1.11
GAIN	44.5	42.4	40.2*	39.7*

[a] : Before standardization (culling)
[b] : After standardization (culling)
* : Statistically different from control, $p \leq 0.05$

Table 5.6.1-12: Mean (SE) male pup weights (g)

F_1 Generation				
Lactation Day	**Control 0 ppm**	**LDT 200 ppm**	**MDT 700 ppm**	**HDT 2000 ppm**
0 S.E	6.2 0.20	6.2 0.22	5.9 0.15	6.2 0.24
4[a] S.E.	10.2 0.53	10.2 0.41	9.6 0.33	10.2 0.48
4[b] S.E.	10.2 0.52	10.1 0.42	9.6 0.25	10.2 0.45
7 S.E.	16.3 0.83	16.4 0.61	15.3 0.33	15.7 0.57
14 S.E.	33.4 0.96	32.3 0.90	30.1 0.75	30.7 0.92
21 S.E.	51.1 1.75	49.1 1.19	46.8 1.43	47.0 1.41

[a] : Before standardization (culling)
[b] : After standardization (culling)

Table 5.6.1-13: Mean (SE) female pup weights (g)

F_1 Generation				
Lactation Day	**Control 0 ppm**	**LDT 200 ppm**	**MDT 700 ppm**	**HDT 2000 ppm**
0 S.E	5.9 0.16	5.9 0.16	5.6 0.11	5.8 0.22
4[a] S.E.	10.1 0.43	9.9 0.35	9.3 0.21	9.8 0.41
4[b] S.E.	10.0 0.45	9.9 0.35	9.3 0.22	9.7 0.40
7 S.E.	16.1 0.62	16.0 0.42	14.7 0.25	14.8 0.38
14 S.E.	32.9 0.79	31.9 0.74	29.7** 0.45	29.0** 0.52
21 S.E.	49.9 1.16	47.9 0.94	45.3** 0.94	44.1** 0.78

[a]: Before standardization (culling)
[b]: After standardization (culling)
*: Statistically different from control, $p \leq 0.05$
**: Statistically different from control, $p \leq 0.01$

3. Offspring postmortem results

Organ weights

Test substance-related organ weight changes were not observed at any dietary level tested. Significant changes in brain weight in the 2000 ppm dose group and slight changes in the 700 ppm dose group were observed (decreased absolute and increased relative) and are considered to be secondary to body weight declines observed in the pups at these same dose groups.

Pathology

Macroscopic examination

There were no test substance-related gross necropsy findings observed at any dietary level tested.

Microscopic examination

Histopathology was not performed for the tissues of the F_1-pups in this study.

III. Conclusions

The parental systemic LOAEL for males is 2000 ppm (147.5 mg BYI 02960/kg bw/day), based on decreased body weight gain. The parental systemic NOAEL for males is 700 ppm (50.1 mg BYI 02960/kg bw/day in males).

The parental systemic LOAEL for females is 700 ppm (60.0 mg BYI 02960/kg bw/day females), based on decreased body weight, decreased body weight gain, alterations in food consumption during premating and decreased spleen weights. The parental systemic NOAEL for females is 200 ppm (17.5 mg BYI 02960/kg bw/day in females).

The reproductive NOAEL is greater than 2000 ppm in males and females (>147.5 mg BYI 02960/kg bw/day in males; >168.9 mg BYI 02960/kg bw/day in females) based on no reproductive findings observed at the highest dose tested.

The offspring LOAEL is 700 ppm (60.9 mg BYI 02960/kg bw/day). The LOAEL is based on maternal effects leading to secondary-mediated effects on pup weight, pup weight gain and organ weight changes (brain). The offspring NOAEL is 200 ppm (17.5 mg BYI 02960/kg bw/day).

Report:	**KIIA 5.6.1 /02; [redacted];2011**
Title:	Technical Grade BYI 02960: A Two-Generation Reproductive Toxicity Study in the Wistar Rat
Report No.: Document No.:	09-R72-SA M-417665-01-2
Guidelines:	OPPTS Guideline Number: 870.3800 Reproduction and Fertility Effects EU Guidelines on Reproductive Toxicity Studies 91/414/EEC; OECD 416 Two-Generation Reproduction Toxicity Study; JMAFF 12 Nousan No. 8147 Health Canada, Guideline on Reproduction Toxicity Studies
Deviations:	No
GLP	Yes

Executive Summary

The principal objective of this reproduction toxicity study was to determine the potential for technical grade BYI 02960 (batch number 2009-000239, a beige powder of 96.2% of purity), administered in the ration, to elicit reproductive and/or developmental effects in the Wistar rat. In this study, BYI 02960 was administered continuously in the food to the Wistar rat (30 animals/dose/sex) at nominal dietary concentrations of 0, 100, 500, and 1800 ppm, dose selections based upon the findings in the range-finder 1 generation reproduction study. All test diets (including control) were available ad libitum for consumption; the homogeneity and stability of BYI 02960 as a dietary admixture was confirmed. Body weight and food consumption determinations and detailed clinical examinations of each animal were conducted weekly throughout the study, as well as an evaluation of multiple reproductive parameters: estrous cycle, sperm counts, motility and morphology, reproductive performance (litter size, mating, fertility and gestation indices, days to insemination and gestation length). In general, animals placed on study were subject to a postmortem examination, which included documenting and saving all gross lesions, weighing designated organs and collecting representative tissue specimens for histopathologic evaluation. Effects attributed to exposure to BYI 02960 were as follows:

<u>At 1800 ppm</u>

P-generation Adults: Females exhibited declines in absolute body weight and body weight gain as well as declines in food consumption on a g/animal/day basis during the premating period. Significant body weight declines were observed in the females throughout gestation. A slight non statistical decline in body weight gain, decreased 9.6% relative to controls, was also noted. Slight non statistical increases in food consumption on a g/kg/day basis were also observed in the females throughout gestation. Females during lactation exhibited significant declines in body weight throughout the lactation period. A significant decline in terminal body weight for females was observed with no test substance-related findings on organ weights. In the males, there were no observed effects on body weight, body weight

gain or food consumption. However, increased absolute and relative liver weights were observed, as well as increased absolute thyroid weights. Minimal centrilobular hypertrophy of the liver was observed in the males and correlated with the increased liver weights.

F_1-Offspring: A significant decline in birth weight was observed (declined 8.2%, relative to controls). Although statistically significant, the birth weight in this dose group did fall within this laboratory's historical control range [Post Natal Day (PND) 0 range 5.5-6.3 grams]. Substantial declines in absolute body weight continued and increased in magnitude by PND 21. An overall decline in pup body weight gain throughout lactation was also observed. A significant delay in preputial separation and a slight nonstatistical delay in vaginal patency were observed in parallel with the decreased pup weight exhibited during lactation in this same dose group. Variations in brain, thymus, and spleen weights in male and/or females were observed and are considered to be due to the decreased body weights observed at this same dietary level and not a direct effect of the test substance.

F_1-generation Adults: In the males, significant declines in body weight were observed beginning on Day 0 and continuing throughout exposure. Food consumption on a g/kg/day basis was increased throughout premating with food consumption on a g/animal/day basis comparable to the control group. Body weight and food consumption observations noted for the males are considered to be a consequence of the body weight effects observed on these animals as pups. For the females, significant declines in body weight and body weight gain were observed beginning Day 0 and continuing throughout the premating period. In the females, significant declines in body weight were observed throughout gestation [Gestation Day (GD) 0-20]. Body weight gain was also declined during the gestation period and occurred concomitantly with the decreased litter size observed in this dose group. Also observed in the females during gestation were significant increases in food consumption on a g/kg/day basis. Females exhibited significant declines in body weight throughout the lactation period, as well as declines in food consumption on a gram/animal/day basis. A significant decline in terminal body weight for males and females was observed.

F_2-Offspring: A slight decrease in litter size was noted in the F_2-generation pups. The decline in litter size (9.2) is just outside of this laboratory's historical control range (9.8-11.8), and declines in total gain during gestation for the F_1-adults occurred concomitantly with this finding. Also, associated with the decrease in litter size was a decline in the total number of implantation sites in the F_1-adults, relative to controls. There was no test substance-related effect observed on the viability of the pups after delivery at any dietary level tested. Pup body weights at birth were comparable to the control group. Pup weight declines were observed beginning on Day 7 (declined 8.1%) and continuing to Day 21 (declined 12.5%). Overall body weight gain for the pups was also decreased. No effect on anogenital distance was observed in the F_2-generation pups. Variations in brain, thymus, and spleen weights in male and/or females were observed and are considered to be due to the decreased body weights observed at this same dietary level and not a direct effect of the test substance.

Reproductive Performance (P and F_1): A significant decrease in the number of estrous cycles was observed for the F_1-females. This finding parallels the significant weight loss observed in the females of this same dose group and generation. Results from the evaluation of vaginal smears in the *P*-generation females did not indicate any test substance-related findings. A decline in the total number of

implantation sites was also observed in the F_1-females. This finding, in the F_1-females, parallels the decreased litter size also observed in this same dose group.

At 500 ppm

P-generation Adults: Females exhibited declines in body weight gain during the premating period.

F_1 -Offspring: No test substance-related findings were observed.

F_1-generation Adults: In the males, slight non statistical declines in body weight were observed beginning Day 0 with transient increases in food consumption on a g-kg/day basis. As with the 1800 ppm dose group food consumption on a g/animal/day basis was comparable to controls. Body weight and food consumption findings observed for the males are considered to be a consequence of the body weight effects observed on these animals as pups. For the females, significant declines in body weights and body weight gains were observed beginning Day 0 and continuing throughout the premating period. In the females, significant declines in body weight were observed throughout gestation (GD 0-20) with no effect on body weight gain. Females exhibited significant declines in body weight throughout the lactation period. A significant decline in terminal body weight for females was observed with no test substance-related findings on organ weights.

F_2-Offspring: Pup body weights at birth were comparable to the control group. Pup weight declines were observed beginning on Day 14 and continuing to Day 21. Overall body weight gain for the pups were also declined. No effect on anogenital distance was observed in the F_2-generation pups. Variations in brain, thymus, and spleen weights in male and/or females were observed and are considered to be due to the decreased body weights observed at these same dietary levels and not a direct effect of the test substance.

Reproductive Performance (P and F_1): No test substance-related findings were observed.

At 100 ppm

P-generation Adults: No test substance-related findings were observed.

F_1-Offspring: No test substance-related findings were observed.

F_1-generation Adults: No test substance-related findings were observed.

F_2-Offspring: No test substance-related findings were observed.

Reproductive Performance (P and F_1): No test substance-related findings were observed.

Conclusions

The parental systemic LOAEL is 1800/500 ppm (119.8/39.2 mg BYI 02960/kg bw/day) in males/females, respectively based on increased liver and thyroid weights, and minimal centrilobular hypertrophy in males, and decreased body weight (premating, gestation, and lactation: F_1), body weight gain (premating; P and F_1), and decreased terminal body weight in F_1 females. The parental systemic NOAEL was 500/100 ppm (32.3/7.8 mg BYI 02960/kg bw/day in males/females, respectively).

The reproductive NOAEL was 500/500 ppm (32.3/39.2 mg BYI 02960/kg bw/day in males/females, respectively) based on decreased estrous cycle number, litter size, and the number of implants observed in the F_1 generation at the highest dietary level tested (LOAEL = 1800/1800 ppm (119.8/140.2 mg BYI 02960/kg bw/day in males/females, respectively).

The offspring LOAEL is 500 ppm (39.8 mg BYI 02960/kg bw/day) based on maternal effects leading to secondary effects on pup weight and organ weight changes in brain, thymus, and spleen. The offspring NOAEL is 100 ppm (7.8 mg BYI 02960/kg bw/day).

I. Materials and Methods

A. Material

1. Test Material: BYI 02960
Description: Beige powder
Lot/Batch: 2009-000239
Purity: 96.2% (Date of analysis: 16 January 2009; Expiration: 16 January 2011)
96.2% (Date of analysis: 14 January 2011; Expiration: 14 January 2013)
CAS: 951659-40-8
Stability of test compound: Stable in the diet over a concentration range of 20-2500 ppm when stored for 7 days at room temperature and following freezer storage for a minimum of 28 days

2. Vehicle control: Acetone
Manufacturer: Fisher Scientific Optima
Lot Numbers (Expiration): 093451 (8 September 2012)
093449 (11 September 2012)
096253 (8 January 2013)
097063 (1 March 2013)
101198 (9 April 2013)

3. Test animals:
Species: Rat
Strain: Wistar Han CRL:WI (HAN)
Age at study initiation: (P) 8-9 weeks
Weight at randomization: Males: 160.2 - 223.6 g; Females: 143.4 - 188.3 g
Source: [redacted], US
Acclimation period: September 21, 2009 (receipt)-September 28, 2009 (released for study)
Diet: Purina Mills Certified Rodent Diet 5002 in "meal" form; The "Certification Profile" for Purina Mills Certified Rodent Diet 5002M provided by the vendor is kept on file at [redacted]. No contaminants were present in the food in sufficient quantities to affect the conduct or results of the study. Available *ad libitum*
Water: Tap water (Kansas City, MO), *ad libitum*

Housing:	Animals were housed individually (except during the mating phase and as noted below for the F_1- and F_2-pups) in suspended stainless steel cages with a deotized cage board in the bedding trays. During gestation and lactation, individual dams (and their litter) were housed in polycarbonate cages with corn-cob bedding.	
Environmental condition:	Temperature:	21.3 to 22.3 °C
	Humidity:	36.15 to 60.44%
	Air changes:	At least 10 changes per hour
	Photoperiod:	Alternating 12-hour light and dark cycles

B. Study Design

1. In-life dates

21 September 2009 to 25 July 2010 at [redacted] USA.

2. Animal assignment and treatment

Each animal was given a temporary identification number via cage card when individually housed at receipt. Following a minimum of six days of quarantine/acclimation, animals were examined by a veterinarian and released for study use. The animals were assigned to either a control or one of three chemically-treated groups using a weight stratification-based computer program [DATATOX (Version rC.10), Instem Computer Systems, P/C, Stone, Staffordshire, England]. Only those animals falling within +/- 20% of the mean for all animals of each gender were placed on study. Once animals were assigned to their dose groups, each rat on study had a microchip (BioMedic Data Systems, Inc., Seaford, DE) subcutaneously implanted on its dorsal surface in the region between its scapulae. At a minimum, the chip was encoded with a unique number, specifying the animal's sex, dose group, cage number, and study affiliation. In addition, a laminated cage card, essentially duplicating the information present on the chip, was attached to the outside of each animal's cage. Pups born alive were identified by tattoo and pups found dead were identified with a marking pen.

Study schedule

One hundred and twenty female and one hundred and twenty male rats were assigned to one of four treatment groups (30 rats/sex/group). Nominal doses of 0, 100, 500 and 1800 ppm BYI 02960 mixed in the diet. Animals were exposed to the treated feed throughout the entire in-life phase of the study. In-life phases include: Premating - 10 weeks; Mating - 14 days; Gestation - approximately 22 days; Lactation - weaning on Day 21. F_1-pups were maintained after weaning for approximately 3 - 4 weeks prior to initiation of the second generation.

Table 5.6.1-14: Animal assignment

Test group	Dose in Diet[a] (ppm)	Animals/Group			
		P Male	P Female	F_1 Male	F_1 Female
Control	0	30	30	30	30
Low (LDT)	100	30	30	30	30
Mid (MDT)	500	30	30	30	30
High (HDT)	1800	30	30	30	30

[a] : Diets were administered from beginning of the study until sacrifice.
LDT : Low-dose tested MDT : Mid-dose tested HDT : High-dose tested

Mating procedure

Males and females were exposed to the test substance for ten weeks prior to mating. Mating was accomplished by co-housing one female with one male for up to 14 consecutive days. During the mating phase, vaginal smears were taken each morning and examined for the presence of sperm and/or internal vaginal plug. Females found to be inseminated were placed in a polycarbonate nesting cage. The day on which insemination was observed in the vaginal smear was designated Day 0 of gestation for that female. In order to evaluate those females that might have been inseminated without exhibiting sperm in the vaginal smear or an internal vaginal plug, all remaining females were placed in polycarbonate nesting cages following the 14-day mating period.

3. Dose selection rationale

Dietary levels of BYI 02960 were selected based upon the preliminary results obtained from a pilot rat reproductive toxicity testing study conducted with the test substance at dietary levels of 0, 200, 700, and 2000 ppm BYI 02960 (M-394208-01-2). In that study, the 2000 ppm dietary level produced clear evidence of toxicity that included statistically lower body weight and body weight gains in females during all phases of the study. Declines in body weight and/or body weight gain were also observed in females at 700 ppm, but to a lesser degree. Pup weight declines were observed (PND 14-21) in both the 700 and 2000 ppm groups and correlate with the body weight loss observed in these dams. Organ weight effects included increased liver weights for males in both the 700 and 2000 ppm dietary groups. Organ weight effects in females were not apparent. There were no effects related to treatment at lower dietary levels.
Based on these interim results, the dietary levels selected for the definitive reproduction toxicity study were 0, 100, 500, and 1800 ppm BYI 02960.

4. Dosage preparation and analysis

The test substance was dissolved in acetone and then mixed with the feed. Treated diet was mixed at room temperature; aliquots of the chemical were taken from the original test batch and transferred to the mixing area. The control test diet was prepared in the same manner as the chemically-treated test diet, excluding only the test substance. A sample of each batch of feed mixed was taken and retained in the freezer until the study was complete and the analytical data deemed satisfactory. Replacement admixtures for each treatment group were prepared weekly (or at greater intervals based on freezer stability) and stored under freezer conditions until presented to the animals the following week (or weeks).

During the lactation phase, a substantial increase in food consumption is observed in all dams which results in greatly increased intake of test substance (normal occurrence). A decrease in the dietary concentration of the test substance offsets this increased food consumption, thereby maintaining an approximately constant test substance intake (mg/kg body weight/day) throughout the study. Accordingly, the concentration of BYI 02960 in the feed for the females only was adjusted during the lactation period (Days 0 - 21) by 50% with one exception. The exception was that during a brief period of the lactation phase for F_1-females, feed was inadvertently not adjusted by 50%. This affected one female in the 100 ppm group on LD7 (for one day) and from 2-10 animals per day for one to three days. After discovering this deviation, the feed was replaced with adjusted feed (as per protocol). The result of this deviation is limited to a brief increase in test substance intake for selected animals during one to three days of the lactation phase. A.I. calculations on the substance intake table for lactation for second generation are based on all animals receiving adjusted feed. Results do not indicate a direct impact on the outcome of the study given the small magnitude of the deviation (relative to the entire phase). Samples from the first batch of adjusted food for each dietary level were analyzed to measure the concentration.

The mean daily intake of the test substance (mg BYI 02960/kg bw/day) throughout this two-generation reproduction study at nominal dietary concentrations of 0, 100, 500 or 1800 ppm, respectively, is summarized in Table 5.6.1-15.

Table 5.6.1-15: Mean daily intake of the test substance

Phase of Study	100 ppm in mg/kg/day[a]	500 ppm in mg/kg/day[a]	1800 ppm in mg/kg/day[a]
Premating (P-gen) - Male	6.6	32.5	117.4
Premating (F_1-gen) - Male	6.4	32.0	122.1
Mean P- and F_1-gen - Male	**6.5**	**32.3**	**119.8**
Premating (P-gen) - Female	7.7	38.7	137.0
Premating (F_1-gen) - Female	7.8	39.6	143.4
Mean P- and F_1-gen - Female	**7.8**	**39.2**	**140.2**
Gestation (P-gen) - Female	6.9	34.3	134.0
Gestation (F_1-gen) - Female	7.0	36.6	168.8
Mean Gestation P and F_1	**7.0**	**35.5**	**151.4**
Lactation (P-gen) - Female	7.8	37.4	140.4
Lactation (F_1-gen) - Female	7.7	42.2	160.3
Mean Lactation P and F_1	**7.8**	**39.8**	**150.4**

[a] : Individual values were based on the means for each particular phase

The concentration of BYI 02960 in the various test diets was verified for batches intended for weeks 1, 2, 3, and at monthly intervals thereafter (Bayer CropScience LP, Residue and Environmental Chemistry Group, [redacted], KS). Test diets intended for the first week of lactation were also analyzed.

Homogeneity Analysis

The mean concentrations of BYI 02960 in the food, sampled from three distinct layers in the mixing bowl and containing a nominal concentration of either 20 or 2500 ppm, were determined to be 19.0 ppm

(range 18.4 - 19.4 ppm; % RSD = 1.83) and 2445 ppm (range 2361 - 2505 ppm; % RSD = 1.6), respectively. Based on a % RSD ≤ to 10%, BYI 02960 was judged to be homogeneously distributed in the food over a concentration range of 20 - 2500 ppm (Moore, 2010).

Stability Analysis

Following 7 days of room temperature storage, the analytically-determine concentration of the AI of the test substance in the 20- or 2500-ppm admixture was determined to be 19.8 ppm (19.2 ppm on Day 0) and 2632 ppm (2445 ppm on Day 0), respectively.

Following 28 days of freezer storage, the analytically-determined concentration of the AI of the test substance in the 20- and 2500-ppm admixtures was determined to be 19.1 ppm (19.0 on Day 0) and 2531 ppm (2445 on Day 0), respectively. BYI 02960 mixed in rodent ration was judged to be stable at room temperature for at least 7 days and following freezer storage for a minimum of 28 days, over a concentration range of 20-2500 ppm (Moore, 2010).

Concentration Analysis

Mean analytical concentrations for each dose group were 99.8, 495, and 1793 ppm, ranging from 99 - 100% of the corresponding nominal concentrations of 100, 500, and 1800 ppm, respectively. During lactation, the concentration of the test substance in the feed for females was adjusted by 50%. Mean analytical concentrations for each dose group during lactation were 49.4, 247, and 902 ppm, ranging from 99 - 100% of the corresponding nominal concentrations of 50, 250, and 900 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 115% and ranged from 104 - 123% for rodent ration spiked with 50, 100, 250, 500, 900, or 1800 ppm of BYI 02960 (Neal, 2011).

5. Statistics and calculation of reproductive and offspring indices

Statistical analyses

Parametric data (including body weight gain and food consumption) were analyzed using a univariate Analysis of Variance (ANOVA), and if significant differences were observed, a Dunnett's Test was performed. Nonparametric data (*e.g.*, number of estrous cycles, litter size, and number of implantation sites) were first analyzed by the Kruskal-Wallis Test and then subjected to Dunn's Test if significant differences were identified. Nonparametric dichotomous data (*e.g.*, fertility and gestation indices) were initially analyzed by the Chi-Square Test and if significance was observed between groups then by the Fisher's Exact Test with the Bonferroni adjustment. Statistical analysis of the gross pathology data was not deemed necessary. Sperm parameters were analyzed using ANOVA (single factor) and the ovarian follicles and corpora lutea count data (mean data animal values) were evaluated by the *t*-Test (Two-sample assuming equal variance test) from Microsoft® Excel® software programs (Microsoft® Office, Excel®, Version 11, USA, 2003, Windows). The organ and terminal body weight data for the adults were evaluated initially using Bartlett's Test to determine homogeneity of variance. If data were homogeneous, an Analysis of Variance (ANOVA) was performed followed by Dunnett's *t*-Test on parameters showing a significant effect by ANOVA. If data were non-homogeneous, a Kruskal-Wallis Analysis of Variance was performed followed by a pair wise Mann-Whitney U Test on parameters showing a significant overall effect. Micropathology data for adult animals were evaluated using the Chi-Square test followed by a one-tailed Fisher's Exact test in cases of significant variation by the Chi-Square analysis. Differences between the control and test substance-treated groups were considered statistically significant when $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$.

Reproductive Indices

The following reproductive indices were calculated from breeding and parturition records of animals in the study:

$$\text{Mating Index (\%)} = \frac{\text{\# inseminated females}^{a}}{\text{\# of females co-housed}} \times 100$$

$$\text{Fertility Index (\%)} = \frac{\text{\# of pregnant females}^{b}}{\text{\# of inseminated females}} \times 100$$

$$\text{Gestation Index (\%)} = \frac{\text{\# of females with live pups}}{\text{\# of pregnant females}} \times 100$$

[a] Includes pregnant females not observed sperm positive or with an internal vaginal plug.

[b] Includes females that did not deliver, but had implantation sites.

Offspring viability indices

The following viability indices were calculated from lactation records of litters in the study:

$$\text{Birth Index (\%)} = \frac{\text{total \# of pups born/litter}}{\text{total \# of implantation sites/litter}} \times 100$$

$$\text{Live birth Index (\%)} = \frac{\text{\# of live pups born/litter}}{\text{total \# of pups/litter}} \times 100$$

$$\text{Viability Index (\%)} = \frac{\text{\# of live pups/litter on day 4 (pre-culling)}}{\text{\# of live pups born/litter}} \times 100$$

$$\text{Lactation Index (\%)} = \frac{\text{\# of live pups/litter on day 21}}{\text{\# of live pups/litter on day 4 (post-culling)}} \times 100$$

Gestation Length = Number of whole days from day in which insemination is observed in the vaginal smear [designated Day 0 of gestation (GD)] to Lactation Day (LD) 0 (delivery of pups and entry in computer system).

C. Methods

1. Parental animals

Mortality and clinical observations

Mortality checks (cage-side observations) were performed twice daily (a.m. and p.m.), during the work week and once daily on weekends and holidays (exceptions listed in Attachment I - Study Protocol and Protocol Variations). Cage-side observations characterized mortality, moribundity, behavioral changes, signs of difficult or prolonged delivery, and overt toxicity by viewing the animal in the cage. In the

event a possible clinical sign was observed during the cage-side evaluation, the animal may have been removed from the cage and a detailed assessment conducted. A detailed evaluation of clinical signs included both observing the animal in the cage and removing the animal to perform a physical examination and was conducted at least once per week throughout the entire in-life phase of the study.

Body weights and food consumption

Body weight and food consumption was measured and fresh feed provided once per week for both males and females during the 10-week premating period. During the mating period and until sacrifice, body weight for the males and unmated females was measured once per week. Also during the mating period, fresh feed was provided for both males and unmated females once each week without measuring food consumption. During gestation, dam body weight was measured and fresh food was provided and food consumption measured on Days 0, 6, 13, and 20. During lactation, dam body weight and food consumption was measured on Days 0, 4, 7, 14, and 21. Fresh food was provided and food consumption measured once per week, with the exception of Week 1 when food consumption was measured twice (Days 0 - 4 and 4 - 7).

Estrous cyclicity

The estrous cycle (determined by examining daily vaginal smears) was characterized for all P- and F_1-generation females, over a three-week period prior to mating. Additionally, the estrous cycle stage was determined for all females just prior to termination.

Sperm parameters

For all P- and F_1-generation males at termination, sperm was collected from one testis and one epididymis for enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively. In addition, an evaluation of the morphology and motility was performed on sperm sampled from the distal portion (closest to the urethra) of the vas deferens. Sperm motility and counts were conducted using IVOS (Integrated Visual Operating Systems, 2005, Hamilton Thorne Research). Sperm motility was conducted for all groups and morphology and testicular counts were conducted on the control and highest dietary groups of both generations. Epididymal counts were conducted on the control and highest dietary groups for the P-generation males and all dietary levels for the F_1-generation males.

2. Litter observations

The following litter observations (X) were made (see Table 5.6.1-16).

Table 5.6.1-16: F_1 / F_2 Litter observations

Observation	Time of Observation (lactation day)					
	Day 0	Day 4	Day 7	Day 14	Day 21	Days (0 - 21)
Number of Live Pups						X
Pup Weight	X	X	X	X	X	
Clinical Observations						X
External alterations	X	X	X	X	X	
Number of Dead Pups						X
Sex of each pup (M/F)						
Preputial Separation	Performed post weaning					
Vaginal Patency	Performed post weaning					

The size of each litter was adjusted on lactation day (LD) 4 to yield, as closely as possible, four males and four females per litter. If the number of male or female pups was less than four, a partial adjustment was made (*e.g.*, three females and five males). No adjustment was made for litters of fewer than eight pups. Adjustments were made by random selection of the pups using software provided by SAS. Culled pups were sacrificed by decapitation. Grossly abnormal pups underwent a gross internal and external examination, and all culled pups were discarded. The F_1- and F_2-pups not culled on LD4 were maintained with the dam until weaning on LD21. On LD21, a sufficient number of randomly selected F_1-pups/sex/litter was maintained to produce the next generation. F_1-pups not selected to become parents of the next generation were sacrificed, examined macroscopically, and had organs weighed. One pup/sex/litter for each generation had tissues collected and evaluated for any structural abnormalities or pathological changes, particularly as they may relate to the organs of the reproductive system.

3. Postmortem observations

Parental animals

All surviving parental males were sacrificed as soon as possible after the last litters were born. Maternal animals were sacrificed following the weaning of their respective litters (LD 21). F_1-adult males were sacrificed after the beginning of the delivery phase for the F_1-females. The animals were subjected to postmortem examinations as follows:

Males were euthanized by carbon dioxide asphyxiation and a gross external examination was performed. Terminal body weights were measured and the recording of all gross pathologic alterations, weighing designated organs, and saving all gross lesions was conducted on all males.

Each dam (P- and F_1-generations) was euthanized by carbon dioxide asphyxiation and a gross external examination was performed. Terminal body weights were measured and the recording of all gross pathologic alterations, weighing designated organs, and saving all gross lesions was conducted on all females. The uterus was excised and the implantation sites, if present, were counted.

Females that were sperm positive and/or had an internal vaginal plug but did not deliver were sacrificed and necropsied after gestation day (GD) 24. Females that were never observed as being inseminated and/or with an internal vaginal plug and did not deliver at least 24 days after the completion of the mating phase, were sacrificed and necropsied. A gross necropsy was performed on these animals as

described above. In addition, patency of the cervical/uterine os in these females was examined via flushing of the uterine horns with 10% buffered formalin.

As summarized in Table 5.6.1-17, the following tissues were collected (X), collected and weighed (XX), and micropathology was performed on those tissues designated with an (O).

Table 5.6.1-17: Tissues list for parental animals

XX	Brain	XXO	Epididymis	XXO	Epididymis Cauda (side not utilized for sperm)
XXO	Pituitary	XO	Coagulating Gland	XO	Lung
XXO	Liver	XXO	Ovary	X	Physical Identifier
XXO	Kidney	XO	Oviduct	XO	Vagina
XXO	Spleen	XXO	Prostate	XO	Cervix
XX	Thyroid	XXO	Seminal Vesicle (with coagulating gland and fluid)	XO	Gross Lesions
XX	Thymus	XXO	Testis		
XXO	Adrenal	XXO	Uterus (with oviduct and cervix)		

Animals found moribund while on study were sacrificed and a gross necropsy performed. Animals found dead were necropsied as soon as possible. Necropsy examinations included those parameters previously described. Pups found dead, stillborn, or terminated in a moribund condition underwent a gross necropsy for possible defects and/or to determine the cause of death.

Offspring

The F_1-offspring not selected as parental animals and all F_2-offspring were sacrificed at 21 days of age. These animals were subjected to postmortem examinations (macroscopic and/or microscopic examination). Any gross lesion was documented and collected.

Pups found dead or terminated in a moribund condition underwent a gross necropsy for possible defects and/or cause of death.

As summarized in table 5.6.1-18, the following tissues from 21 day weanlings were collected (X), collected and weighed (XX), and micropathology was performed on those tissues designated with an (O).

Table 5.6.1-18: Tissues list for offspring

XX	Brain	XXO	Uterus	XO	Testis
XX	Spleen	XO	Ovary	XO	Epididymis
XX	Thymus	XO	Vagina	XO	Prostate
XO	Gross Lesions	XO	Cervix	XO	Coagulating Gland
		XO	Oviduct	XO	Seminal Vesicle

II. Results and discussion

A. Parental animals

1. Mortality and clinical signs

There were no test substance-related mortalities or clinical observations observed during the course of this study at any dietary level tested in either generation.

2. Body weight and food consumption

Male (premating)

P-generation males

No test substance-related findings were observed on body weight or body weight gain during the 10-week premating phase at any dietary level tested. Food consumption was unaffected by treatment at any dietary level tested.

F_1-generation males

In the 1800 ppm dose group, significant declines in body weight were observed beginning on Day 0 (declined 12.1%) and continuing throughout exposure (overall mean decline of 11.2%). Food consumption on a g/kg/day basis in the 1800 ppm dose group was increased throughout premating with food consumption on a g/animal/day basis comparable to the control group. Slight nonstatistical declines were also observed in the 500 ppm dose group beginning Day 0 with transient increases in food consumption on a g/kg/day basis. As with the 1800 ppm dose group, food consumption on a g/animal/day basis was comparable to controls. These body weight and food consumption observations noted in both the 500 and 1800 ppm dose groups are considered to be a consequence of the body weight effects observed on these animals as pups. No test substance-related findings were observed on body weight or food consumption during the 10-week premating period at the 100 ppm dietary level.

Female (premating)

P-generation female

Significant body weight declines were observed in the 1800 ppm dose group by Day 7 (declined 7.4%) and continued throughout the premating period, declined 10.2% by Day 70. A decline in body weight gains were noted in both the 500 and 1800 ppm dose groups, relative to controls, and were declined 20.5 and 42.7%, respectively. Body weight effects were not observed at the 100 ppm dietary level. Significant declines in food consumption on a g/animal/day basis were noted throughout premating (overall mean decline of 9.8%) in the 1800 ppm dose group. There were no test substance-related effects on food consumption in either the 100 or 500 ppm dose groups. Incidental declines in food consumption on a g/kg/day basis were noted during the first week of treatment in both the 500 and 1800 ppm dose groups and were comparable to controls by the second week of premating.

F_1-generation female

Significant declines in body weight were observed in both the 500 and 1800 ppm dose groups beginning Day 0 (declined 4.9 and 14.5%, respectively) and continuing throughout the premating period, overall mean decline of 5.9 and 15.3%, respectively. Bodyweight gain was decreased in both the 500 and 1800 ppm dose groups 16.3 and 21.1%, respectively. There was no effect on body weight or body weight gain observed in the 100 ppm dose group. No test substance-related findings were observed on body weight during the 10-week premating period at the 100 ppm dietary level. Statistically significant declines in food consumption on a g/animal/day basis were observed in the 1800 ppm dose group

throughout premating (overall decline of 11.9%). No test substance-related findings were observed on food consumption during the 10-week premating period at the 100 or 500 ppm dietary levels. Incidental, transient increases in food consumption on a g/kg/day basis were observed in both the 500 and 1800 ppm dose groups and are not considered to be treatment-related.

Reported body weight (males - study duration; females - premating) and selected food consumption results for premating are summarized in Table 5.6.1-19.

Table 5.6.1-19: Mean (S.E.) body weight and food consumption[a]

Observations/Study Week	Dose Group			
	Control 0 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
P-Generation Male				
Mean body weight (g) - Week 14 S.E.	436.0 7.81	436.0 5.96	441.3 7.77	438.4 7.54
Mean weight gain (g) Weeks 1-14	200.8	201.9	200.0	198.0
Mean food consumption (g/animal/day) Weeks 1-10	21.4	21.9	21.9	21.7
Mean food consumption (g/kg/day) Weeks 1-10	65.4	67.2	65.6	65.5
P-Generation Female - Pre-mating				
Mean body weight (g) - Week 10 S.E.	237.4 3.33	235.5 3.09	229.0 2.92	219.3** 2.73
Mean weight gain (g) Weeks 1-10	63.4	61.8	50.4	36.3
Mean food consumption (g/animal/day) Weeks 1-10	16.9	16.0	16.0	14.7**
Mean food consumption (g/kg/day) Weeks 1-10	78.5	77.6	78.1	76.4
F_1-Generation Male				
Mean body weight (g) - Week 14 S.E.	454.5 8.27	456.0 7.51	452.3 8.28	410.8** 7.53
Mean weight gain (g) Weeks 1-14	152.3	152.0	166.2	145.1
Mean food consumption (g/animal/day) Weeks 1-10	23.4	23.8	23.4	22.8
Mean food consumption (g/kg/day) Weeks 1-10	62.2	63.7	64.5	68.8**
F_1-Generation Female - Pre-mating				
Mean body weight (g) - Week 10 S.E.	242.1 3.04	237.3 4.06	224.2** 2.90	203.5** 2.49
Mean weight gain (g) Weeks 1-10	52.1	51.5	43.7	41.2
Mean food consumption (g/animal/day) Weeks 1-10	16.8	16.3	16.3	14.8**
Mean food consumption (g/kg/day) Weeks 1-10	77.7	76.8	79.8	80.7

[a] : Food consumption data represents grand means.

** : Statistically different from control, $p \leq 0.01$

Gestation

P-generation

Significant body weight declines were observed in the 1800 ppm dose group throughout gestation (mean decline GD 0-20 of 10%). A slight nonstatistical decline in body weight gain, declined 9.6% relative to controls, was also noted in this same dose group. Body weight effects considered to be test

substance-related were not observed in the 100 or 500 ppm dietary groups. Slight nonstatistical increases in food consumption on a g/kg/day basis were observed throughout gestation in the 1800 ppm dose group. Test substance-related effects were not observed on food consumption at any other dietary level tested.

F_1-generation
Significant declines in body weight were observed in both the 500 and 1800 ppm dose groups throughout gestation (GD0-20), mean decline of 7.1 and 17.0%, respectively. Body weight gain was also reduced by 18.6% in the 1800 ppm dose group. This decline in body weight gain also correlates with the decreased litter size observed in this dose group. Body weight effects were not observed in the 100 ppm dose group. In the 1800 ppm dose group, significant increases in food consumption on a g/kg/day basis were observed (overall increase of 27.2%). Test substance-related effects were not observed on food consumption at any other dietary level tested.

Reported body weight and selected food consumption results during gestation are summarized in Tables 5.6.1-20 and 5.6.1-21 for P-generation females and F_1-generation females, respectively.

Table 5.6.1-20: Mean (S.E.) body weight and food consumption for P-Generation females during gestation[a]

Observations/Study Week	Dose Group			
	Control 0 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
P- Generation Female - Gestation				
Mean body weight (g) - Day 0 S.E.	238.1 4.27	235.2 3.25	232.2 3.10	214.8** 3.06
Mean body weight (g) - Day 6 S.E.	255.0 3.65	250.2 3.07	248.6 3.22	228.1** 3.25
Mean body weight (g) - Day 13 S.E.	276.0 3.54	272.3 3.57	267.9 3.63	247.1** 3.06
Mean body weight (g) - Day 20 S.E.	333.6 5.68	326.5 4.28	322.1 5.41	301.1** 4.43
Mean weight gain (g) - Days 0-20 S.E.	95.6 3.63	91.3 2.50	89.9 3.74	86.4 2.87
Mean food consumption (g/animal/day) Days 0-20	18.1	17.9	17.9	17.5
Mean food consumption (g/kg/day) Days 0-20	70.6	70.9	71.6	76.2

[a] : Food consumption data represents grand means ** : Statistically different from control, p ≤0.01

Table 5.6.1-21: Mean (S.E.) body weight and food consumption for F_1-Generation females during gestation[a]

Observations/Study Week	Dose Group			
	Control 0 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
F_1-Generation Female - Gestation				
Mean body weight (g) - Day 0 S.E.	243.4 3.27	238.3 4.82	224.4** 3.01	202.6** 2.85
Mean body weight (g) - Day 6 S.E.	257.6 3.09	251.0 4.57	238.7** 2.86	214.0** 3.23
Mean body weight (g) - Day 13 S.E.	277.8 3.33	270.0 4.54	258.3** 2.80	230.8** 2.98
Mean body weight (g) - Day 20 S.E.	335.1 4.52	327.4 5.28	313.8** 4.01	277.2** 4.28
Mean weight gain (g) - Days 0-20 S.E.	91.8 2.39	89.1 2.50	89.4 2.86	74.7** 2.11
Mean food consumption (g/animal/day) Days 0-20	18.2	17.6	17.8	19.0
Mean food consumption (g/kg/day) Days 0-20	70.2	69.7	74.3	89.3**

[a] : Food consumption data represents grand means ** : Statistically different from control, p ≤0.01

Lactation

P-generation

Significant declines in body weight were observed in the 1800 ppm dose group throughout lactation (mean decline LD 0-21 of 9.2%). No effects on body weight were noted in the 100 or 500 ppm dietary levels. No test substance-related effects were observed on food consumption during lactation at any dietary level tested.

F_1-generation

Significant declines in body weight were observed in the 500 and 1800 ppm dose groups throughout lactation (mean decline LD 0-21 of 7.5 and 16.6%, respectively). No effects on body weight were noted in the 100 ppm dietary group. Declines in food consumption were observed on a g/animal/day basis in the 1800 ppm dose group, declined 12.8% (LD 4-21). No test substance-related effects were observed on food consumption during lactation at the 100 ppm dietary level.

Reported body weight and selected food consumption results for lactation are summarized in Table 5.6.1-22.

Table 5.6.1-22: Mean (S.E.) body weight and food consumption for P- and F_1-Generation females during lactation[a]

Observations/Study Week	Dose Group			
	Control 0 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
P-Generation Females - Lactation				
Mean body weight (g) - Day 0	262.8	256.1	250.8	233.3**
S.E.	3.22	2.95	4.30	3.24
Mean body weight (g) - Day 4	271.3	271.6	265.8	245.7**
S.E.	3.12	3.36	3.91	3.66
Mean body weight (g) - Day 7	277.4	276.7	271.2	251.6**
S.E.	2.81	3.05	3.90	3.20
Mean body weight (g) - Day 14	292.6	292.3	287.5	267.1**
S.E.	3.81	2.77	4.22	3.17
Mean body weight (g) - Day 21	285.8	281.4	280.1	264.3**
S.E.	3.67	2.80	3.59	3.15
Mean food consumption (g/animal/day) Days 0-21	41.5	43.1	41.6	40.2
Mean food consumption (g/kg/day) Days 0-21	149.2	155.9	153.3	159.8
F_1-Generation Females - Lactation				
Mean body weight (g) - Day 0	263.0	254.5	242.5**	218.4**
S.E.	3.38	4.53	3.27	2.84
Mean body weight (g) - Day 4	277.3	265.9	256.0**	225.1**
S.E.	3.24	4.13	3.51	3.09
Mean body weight (g) - Day 7	278.7	268.5	260.9**	231.3**
S.E.	3.23	4.02	3.43	3.02
Mean body weight (g) - Day 14	294.8	282.8	272.2**	244.3**
S.E.	3.54	3.64	3.72	3.53
Mean body weight (g) - Day 21	283.8	278.5	261.6**	247.1**
S.E.	3.45	3.91	4.17	3.22
Mean food consumption (g/animal/day) Days 0-21	45.3	42.5	44.0	40.1
Mean food consumption (g/kg/day) Days 0-21	162.0	158.5	169.4	173.2

[a] : Food consumption data represents grand means ** : Statistically different from control, p ≤0.01

3. Test substance intake

Based on food consumption, body weight and dietary analyses results, the doses expressed as mean daily mg test substance/kg body weight during the pre-mating period (10 weeks for males and females) are presented in Table 5.6.1-23.

Calculation for test substance intake is:
[Mean analytical concentration (ppm) specific for premating/1000] X mean weekly food consumption (g/kg/body weight/day) during premating.

Table 5.6.1-23: Test substance intake during premating (mg/kg body weight/day)

	Male			Female		
	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
P	6.6	32.5	117.4	7.7	38.7	137.0
F_1	6.4	32.0	122.1	7.8	39.6	143.4
Mean of both Generations	**6.5**	**32.3**	**119.8**	**7.8**	**39.2**	**140.2**

4. Reproductive function

Estrous cycle length and periodicity

In the 1800 ppm dose group, a significant decrease in the number of estrous cycles was observed for the F_1-females. This finding parallels the significant weight loss observed in the females of this same dose group and generation. Results from the evaluation of vaginal smears in the P-generation females did not indicate any test substance-related findings at any dietary level tested.

The estrous cycle data for P- and F_1-generation females is summarized in Table 5.6.1-24.

Table 5.6.1-24: Estrous cycle length and periodicity in P- and F_1-Generation females

Observation	Dose Group (ppm)			
	Control 0 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
P-Generation				
Number of Estrous Cycles (S.E.)	3.5 (0.1)	3.7 (0.1)	3.4 (0.2)	3.4 (0.1)
Estrous Cycle Length (S.E.)	4.4 (0.3)	4.3 (0.1)	4.3 (0.2)	4.3 (0.1)
F_1-Generation				
Number of Estrous Cycles (S.E.)	3.5 (0.2)	3.3 (0.2)	3.3 (0.2)	2.9* (0.2)
Estrous Cycle Length (S.E.)	4.0 (0.2)	4.1 (0.1)	4.4 (0.2)	4.4 (0.1)

* : Statistically different from control $p \leq 0.05$

Sperm measures

There were no effects considered to be test substance-related on any sperm parameter evaluated at any dietary level tested for either generation. These data are summarized below in Table 5.6.1-25.

Table 5.6.1-25: Sperm measures in P- and F_1-Generation males

Sperm Analysis		Dose Group (ppm)			
		Control 0 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
P Generation Male					
Sperm Motility	% Motile	92.4	90.9	93.3	90.7
	% Progressive	62.1	60.7	63.3	59.2
Sperm Counts (sperm/gram)	Testis	26.9	N/A	N/A	23.7
	Epididymis	158.8	N/A	N/A	138.7
Sperm Morphology (mean total number)	Normal	193.2	N/A	N/A	197.0
	Abnormal	2.7	N/A	N/A	2.2
	Detached Head	4.1	N/A	N/A	0.8
F_1-Generation Male					
Sperm Motility	% Motile	90.7	94.5	92.0	92.7
	% Progressive	64.0	66.4	64.8	63.3
Sperm Counts (sperm/gram)	Testis	29.4	N/A	N/A	30.9
	Epididymis	158.8	N/A	N/A	141.8
Sperm Morphology (mean total number)	Normal	189.1	N/A	N/A	195.2
	Abnormal	3.8	N/A	N/A	4.2
	Detached Head	0.4	N/A	N/A	0.6

N/A : Not Applicable - evaluation deemed unnecessary

5. Reproductive performance

In the 1800 ppm dose group for the F_1-adults, a decline in the total number of implantation sites was observed. This finding parallels the decreased litter size also observed in this same dose group and generation. Overall reproductive performance was not affected for any other parameter (*e.g.*, mating, fertility or gestation indices, days to insemination, or gestation length) in either generation at any dietary level tested. The significance noted on the median insemination length in the 100 ppm dose group of the F_1-females is considered incidental to treatment as no dose-response relationship exists for this finding.

Results for reproductive performance for both the P- and F_1-generation animals are summarized below in Table 5.6.1-26.

Table 5.6.1-26: Reproductive performance in P- and F_1-Generation animals

Observation	Dose Group (ppm)			
	Control 0 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
P-Generation - F_1-Offspring				
Number Cohoused	30	30	30	30
Number Mated	30	29	29	30
Number of Animals Delivered	29	27	28	28
Number of Animals with Implants	29	27	28	28
Mating Index	100.0	96.7	96.6	100.0
Fertility Index	96.7	93.1	96.6	93.3
Gestation Index	100.0	100.0	100.0	100.0
Mean Number Days to Insemination (S.E.) Median	3.4 (0.67) 3.0	3.3 (0.59) 3.0	3.2 (0.52) 3.0	3.1 (0.59) 3.0
Mean Gestation Length (days) (S.E.) Median Gestation Length (days)	22.1 (0.11) 22.0	22.1 (0.12) 22.0	22.1 (0.12) 22.0	22.0 (0.12) 22.0
Total number of implantation sites (Median)	311 (11.0)	285 (11.0)	298 (10.5)	289 (10.0)
F_1-Generation - F_2-Offspring				
Number Cohoused	30	30	30	30
Number Mated	29	30	29	30
Number of Animals Delivered	27	28	28	29
Number of Animals with Implants	27	28	28	29
Mating Index	96.7	100.0	96.7	100.0
Fertility Index	93.1	93.3	96.6	96.7
Gestation Index	100.0	100.0	100.0	100.0
Mean Number Days to Insemination (S.E.) Median	2.3 (0.23) 2.0	3.7 (0.45) 4.0**	2.6 (0.24) 2.5	2.1 (0.18) 2.0
Mean Gestation Length (days) (S.E.) Median Gestation Length (days)	22.0 (0.09) 22.0	22.1 (0.15) 22.0	21.9 (0.09) 22.0	22.0 (0.08) 22.0
Total number of implantation sites (Median)	305 (12.0)	314 (11.0)	323 (11.0)	281 (10.0**)

** : Statistically different from control p < 0.01

6. Parental postmortem results

Terminal body weight and organ weights

Male

P-generation

Test substance-related effects on terminal body weight were not observed in the males at any dietary level tested in the P-generation. In the 1800 ppm dose group, increased absolute and relative liver weight was observed, increased 9% relative to controls. Also observed in this same dose group were increased absolute thyroid weights, increased 13% in left thyroid and increased 21% in right thyroid, relative to controls. Organ weight changes considered to be test substance-related were not observed in the 100 or 500 ppm dietary groups.

F_1-generation
A significant decline in terminal body weight for males was observed in the 1800 ppm dose group, declined 10% relative to controls. There were no terminal body weight effects observed at any other dietary level tested. Organ weight changes considered to be test substance-related were not observed at any dietary level tested.

Female
P-generation
A significant decline in terminal body weight for females was observed in the 1800 ppm dose group, declined 8% relative to controls. There were no terminal body weight effects observed at any other dietary level tested. Organ weight changes considered to be test substance-related were not observed at any dietary level tested.

F_1-generation
A significant decline in terminal body weight for females was observed in both the 500 and 1800 ppm dose groups, declined 6% and 12%, respectively, relative to controls. Terminal body weight effects were not observed at the 100 ppm dietary level. There were no organ weight changes considered to be test substance-related at any dietary level tested.

Pathology
Macroscopic examination
Test substance-related gross necropsy findings were not observed at any dietary level tested in either generation.

Microscopic examination
In the P-generation males of the 1800 ppm dose group, minimal centrilobular hypertrophy of the liver was observed and correlated with the increased liver weights observed in this same dose group. Test substance-related microscopic findings were not observed in males at any other dietary level tested. Females of the P-generation did not exhibit any test substance-related microscopic findings at any dietary level tested.
Test substance-related microscopic findings were not observed in F_1-males or females at any dietary level tested.

Ovarian follicle counts from F_1-generation
There was no test substance-related effect observed on the mean primordial (preantral) follicles, antral follicles, or corpora lutea counts for the F_1-females at any dietary level tested.

B. Offspring

1. Viability and clinical signs
In the 1800 ppm dose group, a slight decrease in litter size was noted in the F_2-generation pups. The decline in litter size (9.2) is just outside of this laboratory's historical control range (9.8 - 11.8) and declines in total gain during gestation for the F_1-adults occurred concomittently with this finding. Also, associated with the decrease in litter size was a decline in the total number of implantation sites in the

F_1-adults, relative to controls. There was no test substance-related effect observed on the viability of the pups after delivery at any dietary level tested. Test substance-related clinical observations were not observed in either generation at any dietary level tested.

Mean litter size and pup viability (survival) during lactation are summarized in Table 5.6.1-27.

Table 5.6.1-27: Litter parameters from pups during lactation

Observation	Dose Group (ppm)			
	Control 0 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
P Generation				
Total number of pups born	297	277	282	281
Number stillborn	0	2	0	0
Sex Ratio Day 0 (% male)	55.1	53.0	54.7	49.6
Mean litter size Day 0 Median	10.2 11.0	10.3 11.0	10.1 10.0	10.0 10.0
Birth index	95.0	97.1	93.3	97.4
Live birth index	100.0	99.0	100.0	100.0
Viability index	98.0	99.3	99.7	98.1
Lactation index	99.1	99.5	100.0	99.1
F_1-Generation				
Total number of pups born	291	300	311	266
Number stillborn	1	0	1	4
Sex Ratio Day 0 (% male)	51.6	50.6	50.6	47.7
Mean litter size Day 0 Median	10.8 11.0	10.7 11.0	11.1 10.5	9.2 **10.0***
Birth index	95.6	92.7	96.4	94.8
Live birth index	99.7	99.7	99.2	98.2
Viability index	99.3	99.7	99.4	97.2
Lactation index	99.1	100.0	100.0	98.7

* : Statistically different from control, p ≤ 0.05

2. Pup body weight (combined male and female)

F_1-Pups: A significant decline in birth weight was observed in the 1800 ppm dose group (declined 8.2%, relative to controls), but the birth weight in this dose group did fall within this laboratory's historical control range (PND 0 range 5.5-6.3 grams). Birth weight was not affected at the other dietary levels tested. Significant declines in absolute body weight continued in the 1800 ppm dose group, declined 8.8% by PND 4, and these declines increased in magnitude to 13.2% by PND 21. An overall decline in body weight gain throughout lactation was also observed in the 1800 ppm dose group (decline in gain of 13.7%). Body weight effects were not observed at the 100 or 500 ppm dietary levels.

F_2-Pups: Pup body weights at birth for all three treated groups were comparable to the control group. Pup weight declines were observed in both the 500 and 1800 ppm dose groups. In the 500 ppm dose

group, pup weight declines were observed beginning on Day 14 (declined 6.9%) and continuing to Day 21 (declined 7.4%). In the 1800 ppm dose group, pup weight declines were observed beginning on Day 7 (declined 8.1%) and continuing to Day 21 (declined 12.5%). Overall body weight gain for the pups of the 500 and 1800 ppm dose groups were declined 7.7 and 13.8%, respectively. There were no test substance-related effects on pup body weight or body weight gain observed during the lactation period at the 100 ppm dietary level.

Selected mean pup body weight data are presented in Tables 5.6.1-28, 5.6.1-29, and 5.6.1-30 for combined sexes, and male and female pups individually, respectively.

Table 5.6.1-28: Mean (S.E.) combined male and female pup weights (g)

F_1-Generation					F_2-Generation				
Lactation Day	**Control 0 ppm**	**LDT 100 ppm**	**MDT 500 ppm**	**HDT 1800 ppm**	**Lactation Day**	**Control 0 ppm**	**LDT 100 ppm**	**MDT 500 ppm**	**HDT 1800 ppm**
0	6.1	6.0	5.9	5.6**	**0**	6.1	6.0	5.9	5.8
S.E	0.11	0.11	0.11	0.11	**S.E**	0.10	0.10	0.10	0.08
4[a]	10.2	10.3	9.8	9.3*	**4[a]**	10.1	9.8	9.6	9.6
S.E.	0.26	0.25	0.28	0.27	**S.E.**	0.23	0.21	0.25	0.22
4[b]	10.3	10.3	9.8	9.3*	**4[b]**	10.1	9.7	9.7	9.7
S.E.	0.26	0.25	0.28	0.26	**S.E.**	0.23	0.21	0.25	0.22
7	16.1	16.1	15.3	14.2**	**7**	16.0	15.6	15.3	14.7*
S.E.	0.32	0.35	0.40	0.36	**S.E.**	0.32	0.23	0.36	0.38
14	32.3	31.9	30.6	28.0**	**14**	32.1	31.2	29.9*	27.9**
S.E.	0.46	0.61	0.70	0.49	**S.E.**	0.60	0.41	0.51	0.75
21	49.3	48.9	47.3	42.8**	**21**	48.8	47.4	45.2**	42.7**
S.E.	0.63	0.88	1.00	0.69	**S.E.**	0.77	0.72	0.72	1.10
GAIN	43.2	42.9	41.4	37.3**	**GAIN**	42.7	41.5	39.4**	36.8**

[a] : Before standardization (culling); [b] : After standardization (culling);
* : Statistically different from control, $p \leq 0.05$ ** : Statistically different from control, $p \leq 0.01$

Table 5.6.1-29: Mean (S.E.) male pup weights (g)

F_1-Generation					F_2-Generation				
Lactation Day	**Control 0 ppm**	**LDT 100 ppm**	**MDT 500 ppm**	**HDT 1800 ppm**	**Lactation Day**	**Control 0 ppm**	**LDT 100 ppm**	**MDT 500 ppm**	**HDT 1800 ppm**
0	6.2	6.2	6.0	5.7**	**0**	6.2	6.0	6.0	6.0
S.E	0.11	0.11	0.10	0.12	**S.E**	0.10	0.11	0.11	0.08
4[a]	10.4	10.4	9.9	9.4*	**4[a]**	10.3	9.7	9.8	9.8
S.E.	0.24	0.24	0.28	0.29	**S.E.**	0.23	0.23	0.26	0.22
4[b]	10.4	10.4	9.9	9.4*	**4[b]**	10.3	9.7	9.8	9.8
S.E.	0.25	0.24	0.28	0.29	**S.E.**	0.23	0.22	0.26	0.21
7	16.2	16.3	15.5	14.4**	**7**	16.3	15.8	15.5	15.0
S.E.	0.30	0.34	0.42	0.39	**S.E.**	0.33	0.29	0.38	0.40
14	32.5	32.1	30.8	28.2**	**14**	32.4	31.5	30.3*	28.4**
S.E.	0.42	0.62	0.70	0.53	**S.E.**	0.64	0.48	0.51	0.76
21	49.8	49.3	48.0	43.5**	**21**	49.6	48.1	45.9**	43.8**
S.E.	0.89	0.96	1.00	0.78	**S.E.**	0.80	0.86	0.74	1.19

[a] : Before standardization (culling); [b] : After standardization (culling);
* : Statistically different from control, $p \leq 0.05$ ** : Statistically different from control, $p \leq 0.01$

Table 5.6.1-30: Mean (S.E.) female pup weights (g)

F_1-Generation					F_2-Generation				
Lactation Day	Control 0	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm	Lactation Day	Control 0 ppm	LDT 100 ppm	MDT 500 ppm	HDT 1800 ppm
0	5.9	5.9	5.7	5.4*	**0**	5.9	5.8	5.7	5.6
S.E	0.12	0.12	0.11	0.11	**S.E**	0.11	0.09	0.10	0.08
4[a]	10.0	10.1	9.7	9.1	**4[a]**	9.9	9.6	9.4	9.5
S.E.	0.28	0.28	0.29	0.27	**S.E.**	0.24	0.21	0.24	0.25
4[b]	10.0	10.1	9.7	9.1	**4[b]**	9.9	9.6	9.4	9.5
S.E.	0.29	0.28	0.29	0.26	**S.E.**	0.24	0.21	0.24	0.25
7	15.8	15.8	15.1	14.0**	**7**	15.5	15.4	15.0	14.4*
S.E.	0.36	0.39	0.41	0.35	**S.E.**	0.34	0.22	0.35	0.39
14	31.9	31.3	30.0	27.7**	**14**	31.8	30.9	29.6*	27.5**
S.E.	0.52	0.60	0.71	0.51	**S.E.**	0.57	0.40	0.52	0.76
21	48.4	47.8	46.1	42.2**	**21**	47.8	46.8	44.4**	41.8**
S.E.	0.72	0.80	1.05	0.70	**S.E.**	0.77	0.71	0.74	1.07

[a] : Before standardization (culling); [b] : After standardization (culling);
* : Statistically different from control, $p \leq 0.05$; ** : Statistically different from control, $p \leq 0.01$

3. Pup sexual maturation

In the high dose group (1800 ppm) a significant delay in preputial separation and a slight nonstatistical delay in vaginal patency were observed and are considered to be a consequence of the decreased pup weight exhibited during lactation in this same dose group rather than an endocrine-mediated mode of action. This explanation is supported by the similar mean body weight values on the day of complete PPS between controls (178 g) and the 1800 ppm group (180g). Similarly, the apparent delay in vaginal patency (by 1 day) in the 1800 ppm group is strongly correlated to decreased body weight and body weight gain during lactation as well as decreased maternal body weight parameters throughout the study. Additional support is seen in the lack of treatment-related effects on other endocrine-sensitive parameters in both males and females. There were no effects observed on either vaginal patency or balanopreputial separation at any other dietary level tested.

4. Pup anogenital distance

There was no effect observed on anogenital distance at any dietary level tested for either the males or females of the F_2-generation.

5. Offspring postmortem results

Organ weights

There were no test substance-related organ weight changes observed for the F_1- or F_2-pups at any dietary level tested. Variations in brain, thymus, and spleen weights in male and/or females of the 500 (F_2) and 1800 ppm (F_1/F_2) dose groups were considered to be due to the decreased body weights observed at these same dietary levels and not a direct effect of the test substance.

Pathology

Macroscopic examination

There were no test substance-related gross necropsy findings observed at any dietary level tested in either the F_1- or F_2-pups.

Microscopic examination

There were no test substance-related microscopic findings observed at any dietary level tested in either the F_1- or F_2-pups.

III. Conclusions

The parental male systemic LOAEL is 1800 ppm (119.8 mg BYI 02960/kg bw/day), based on increased liver weights with centrilobular hypertrophy (minimal) noted upon microscopic exam and increased thyroid weights (absolute/relative). The parental male systemic NOAEL is 500 ppm (32.3 mg BYI 02960/kg bw/day).

The parental female systemic LOAEL is 500 ppm (39.2 mg BYI 02960/kg bw/day), based on decreased body weight (F_1) during premating, gestation, and lactation, and decreased body weight gain (P and F_1) during premating. Also observed were terminal body weight declines for the F_1-females. The parental female systemic NOAEL is 100 ppm (7.8 mg BYI 02960/kg bw/day).

The reproductive LOAEL is 1800 ppm in males and females (119.8 mg BYI 02960/kg bw/day in males; 140.2 mg BYI 02960/kg bw/day in females) based on a decrease in cycle number, a slight decrease in litter size in the F_1-generation, and a decrease in the total number of implantation sites in the F_1-females. The reproductive NOAEL is 500 ppm for both males and females (32.3 mg BYI 02960/kg bw/day in males; 39.2 mg BYI 02960/kg bw/day in females).

The offspring LOAEL is 500 ppm (39.8 mg BYI 02960/kg bw/day). The LOAEL is based on decreased F_2-pup weight and F_2-pup weight gain, and secondary mediated organ (weight changes (brain, thymus, and spleen). The offspring NOAEL is 100 ppm (7.8 mg BYI 02960/kg bw/day).

Report:	**KIIA 5.6.1/03, [redacted]D et al., 2014**
Title:	Flupyradifurone (BYI 02960): Mode of Action implicated in the findings observed in the rat 2 generation reproduction study with Flupyradifurone (M-417665-02-1)
Report No & Document No	M-496018-01-1
Guidelines:	NA
GLP	None

This position paper has been written to clarify the Mode of Action which might account for the offspring reproductive findings seen at the high dose level (i.e. delay in preputial separation (PPS) and vaginal patency (VO); reduced brain, thymus and spleen weights; and reduced number of implantation sites).

Effects unrelated to the endocrine system observed after chemical treatment often confound the interpretation of results of endpoints evaluated in standard reproductive toxicology studies ([redacted] et al., 2004; [redacted] et al., 2007; [redacted] et al., 2007; [redacted] et al., 2000; [redacted] et al., 2000a; for review see [redacted] et al., 2000b). For example, significant body weight loss, altered feed intake, and stress responses can contribute to changes in study endpoints that may be inaccurately attributed to an endocrine-mediated mechanism. Several peer-reviewed publications report studies that have been conducted utilizing food restriction, resulting in varying degrees of BW loss, to determine the influence of BW reduction on *in utero* and postnatal developmental endpoints (Carney et a., 2004; [redacted] et al.,

1993; [redacted] et al., 2000; [redacted] et al., 2007; [redacted] at al., 2007; [redacted] et al., 2000a). Endocrine-related endpoints evaluated in these studies include PPS and sperm parameters in males, VO and estrous cyclicity in females, and organ weights and hormone concentrations in both sexes.

In the rat two-generation reproduction study conducted on Flupyradifurone ([redacted], 2011), changes were observed in offspring at the high dietary concentration of 1800 ppm. Effects seen on PPS and VO, two well-established markers of pubertal development, included a statistically significant delay (PPS) and a slight, non-statistical delay (VO) in F_1 generation animals. In addition, variations in brain, thymus, and spleen weights in offspring of the 500 ppm (F_2 generation) and 1800 ppm (F_1 and F_2 generations) dose groups were also observed. A slight, albeit significant, decrease in median implantation sites (and thus decrease in litter size) in the F_1-generation was observed with 1800 ppm Flupyradifurone. It is the position of both Bayer CropScience (BCS) and the performing laboratory that the aforementioned effects are likely a result of treatment-induced reductions in BW and BWG rather than a direct, endocrine-mediated MOA.

Preputial Separation

Toxicity testing guidelines for the rat two-generation reproduction toxicity study require that systemic toxicity is evident at the highest dose level, and dose levels should be chosen with the aim to induce some reproductive and/or systemic toxicity, but not death or severe suffering (OECD 416; OCSPP 870.3200). As such, effects on BW (reductions >10%) are the most common effects noted at maximum tolerated dose levels, and it is reasonable that delays in preputial separation may be observed as a result of general systemic toxicity rather than a consequence of an endocrine-mediated MOA ([redacted] et al., 2012). In the present study, animals exposed to 100 ppm or 500 ppm Flupyradifurone achieved PPS in a time latency similar to concurrent controls and well within historical control data for Wistar Han rats in the performing laboratory. At the high dietary level of 1800 ppm, however, a statistically significant delay in PPS (3.9 days) was observed compared to controls. The observed delay in PPS in animals at 1800 ppm is likely due to significant BW loss and food consumption and not a result of an endocrine MOA associated with delayed PPS.

A recent study concluded that while treatment-related delays in PPS may be indicative of specific anti-androgenic activity, impaired growth could also alter the onset of puberty ([redacted] et al., 2014). The mean BW at the day of complete PPS was similar between the control animals (178 g) and 1800 ppm group (180 g), however, the mean BW at PND 21 was significantly decreased (-12.5%) compared to control animals; BW at PND 21 is often used as a covariate for evaluating the age at complete PPS ([redacted] et al., 2000b). This significant decrease in BW at PND 21 was accompanied by significant decreases observed in maternal BW of the 1800 ppm group throughout gestation (GDs 0, 6, 13, and 20; see Appendix 3). As other sensitive anti-androgenic parameters investigated in this study such as male fertility, male reproductive organs (weights and histopathology) and sperm parameters (count, motility, or progressive sperm) were not affected at any dietary level of Flupyradifurone, it is likely that the effect on PPS was due to a non-specific effect linked to overt toxicity with decreased BW and retarded growth.

By comparison, direct-acting anti-androgens do not inhibit food consumption or slow growth ([redacted] et al., 1995), but they delay PPS and reduce the weights of the ventral prostate, seminal vesicle, epididymides, Cowper's glands, and levator ani plus bulbocavernosus muscles ([redacted], 2000b; [redacted]

et al., 2006). Compounds that act as anti-androgens may also delay PPS via hypothalamic-pituitary or steroid hormone synthesis disruptions (for review see [redacted] et al., 2000b), but in these cases, changes in testicular weights, testosterone and/or luteinizing hormone concentrations (not evaluated in the current study), and changes in accessory sex gland tissue weights would have accompanied the observed delay in PPS. Furthermore, anti-androgens such as vinclozolin, can decrease male pup anogenital distance (AGD) to an extent similar to female pups ([redacted] et al., 2001). None of these anti-androgenic changes were observed in the two-generation reproduction study or the rat developmental neurotoxicity study ([redacted], 2012) conducted on Flupyradifurone.
These data strongly support the conclusion that the delay in PPS observed in F_1-generation males after exposure with high dietary concentrations of Flupyradifurone is due to significant BW loss and reduced food consumption and BWG over the exposure period, and not due to an endocrine-mediated MOA.

Vaginal Patency

F_1-generation females showed a slight, non-significant delay in VO (1.0 day) in animals in the 1800 ppm dietary concentration group. The mean for complete VO was 34.8 days for the control females and 35.8 days for the high dietary level females. It should be noted, however that the day means to complete VO were within the historical control range (33.4 – 36.7 days) for all dietary levels of Flupyradifurone. The F_1-generation females achieved VO with significantly reduced BW and BWG (-16.6% and -19%, respectively compared to controls) during lactation, and no other disruptions in estrogen sensitive endpoints (i.e. tissue weights, estrous cyclicity, histopathological analysis, mating parameters) were observed in the remainder of the study. Furthermore, no other estrogen sensitive parameters were affected by Flupyradifurone in the rat developmental neurotoxicity study ([redacted], 2012), the developmental toxicity studies ([redacted], 2010; [redacted], 2012), or the chronic toxicity/carcinogenicity studies ([redacted], 2012; [redacted], 2012). Therefore, the slight delay of 1 day in VO after high dietary exposure to Flupyradifurone is most likely the result of decreased BW and BWG during lactation, as well as decreased maternal BW and BWG throughout premating, gestation, and lactation. Delays in VO, concurrent with observations of delayed PPS in males, is understood to indicate a general growth delay, rather than an endocrine MOA ([redacted] et al., 2012). A slight, but statistical, decrease in the number of estrous cycles in the F_1-generation was observed, but is also attributable to BW loss, as irregular cycles are often noted in the immediate post-pubertal period ([redacted] et al., 1985). Taken together, these points indicate that the non-statistically significant delay VO is not endocrine-mediated and is of no biological significance.

Organ Weight Changes in Pups

Organ weight changes in pups were observed in brain, thymus, and spleen in pups exposed to 1800 ppm Flupyradifurone. Similar to changes observed in PPS and VO, the pup organ weight changes were due to significant BW loss over the exposure period, and not due to an endocrine MOA. In F_1-generation pups, birth weight was significantly decreased in both sexes, and an overall decrease in BWG throughout lactation was observed in F1- (-13.7%) and F2-generation (-13.8%) pups at the high dietary level. Pup terminal BWs were also decreased in males (-10%) and females (-12%). In both sexes and generations, absolute brain weights were decreased, while brain weight relative to BW was increased. A similar trend was observed thymus weights, while absolute spleen weights were decreased in F_1 males and both sexes in the F_2 generation.

The organ weight changes and reduction in pup terminal BWs seen in the present study are consistent with changes in the brain, thymus, and spleen observed in a food restriction reproduction study in which animals were given 30% less food daily relative to control groups ([redacted] et al., 2004). Animals receiving 30% less food (equivalent to 6-19% BW decrease) showed similar reductions in absolute organ weights with concomitant increases in relative organ weights in both male and female rats ; animals receiving only 10% less food, which did not change BW, showed no organ weight changes compared to control groups ([redacted] et al., 2004). Animals receiving 30% less food exhibited BW reductions similar in magnitude to those found in animals exposed to the high dietary level of Flupyradifurone in the two-generation reproduction study. These data support the notion that significant reductions in BW can influence organ weights. In addition, no microscopic changes were observed in the brain, thymus, or spleen in animals of either sex in the Flupyradifurone two-generation reproduction study. The rat DNT study ([redacted], 2012) and rat subchronic neurotoxicity study ([redacted], 2011) conducted with Flupyradifurone did not produce any brain weight changes up to the high dietary levels of 1200 ppm and 2500 ppm, respectively. In both studies, BW and BWG declines were not as severe in magnitude as those seen in the two-generation reproduction study, supporting the conclusion that the organ weight changes observed in the reproduction study are likely due to reductions in BW and BWG. In a rat immunotoxicity study conducted with Flupyradifurone ([redacted], 2011), significant decreases in the absolute weight of the spleen and thymus were observed, but relative weights were unchanged for both organs. The lack of response in endocrine-sensitive tissues in both sexes further supports these organ weight changes are due to significant decreases in BW and not an endocrine-mediated event.

Reduced Number of Implantation Sites

A slight, albeit significant, decrease in median implantation sites (and thus decrease in litter size) in the F_1-generation was observed with 1800 ppm Flupyradifurone. This effect, however, was not observed in the P_0-generation dams, and the total number of implantation sites between the P_0- and F_1-generations in the control (311 and 305, P_0 and F_1, respectively) and high dose (289 and 281, P_0 and F_1, respectively) are similar. This slight decrease is likely due to the decreased BW in the P_0 females, which was observed as early as day 7 after initiation of exposure. Significant BW decreases were maintained for the duration of the study, including the F_1 females at birth, and continuing through mating and gestation of the F_2 animals. Exposure to exogenous chemicals that produce a reduction in the amount of BW gained during the first 9 days of pregnancy is an indication of the inherent toxicity of the chemical and may contribute to maternal toxicity and pregnancy loss ([redacted] et al., 2000). No other effects were noted in female organ weights or histopathology in the two-generation reproduction study. Developmental toxicity studies in rats ([redacted], 2010) and rabbits ([redacted], 2012) for Flupyradifurone did not show any significant decreases in the number of implantation sites in dams that showed a significant decrease in BW, nor in any other changes in reproductive indices, tissue weights, or histopathology. Taken together, the data supports that this finding was due to a decreased BW and not an endocrine-mediated MOA.

Summary and Conclusion

The two-generation reproduction study conducted in rats with Flupyradifurone reported changes in a few endpoints that are known to be influenced by endocrine MOAs. Thorough evaluation of the

available data on Flupyradifone and the peer-reviewed literature confirmed the initial position of BCS and the performing laboratory that these changes were secondary to decreases in BW and BWG. Several publications reporting studies that utilized food restriction to evaluate the effect of reduced BW and BWG on developmental and reproductive endpoints support this position. The mammalian toxicity database for Flupyradifurone does not indicate any anti-androgenic or anti-estrogenic activity, two known endocrine-mediated MOAs. Thus, the changes seen in the endpoints in question appear to occur secondary to overt toxicity associated with exposure to Flupyradifurone as evidenced by reductions in BW and BWG throughout all stages, including critical periods of development, of the two-generation reproduction study.

KIIA 5.6.2 - Separate male and female studies

Results of the two-generation study did not show any sex-related findings. Therefore, specific studies on males and females were not triggered.

KIIA 5.6.3 - Three segment designs

In the two-generation study, no effects on reproductive parameters occurred. Therefore, this type of study is not required.KIIA 5.6.4 - Dominant lethal assay for the male fertility

In the two-generation study, no effects on reproductive parameters occurred. Therefore, this type of study is not required.

KIIA 5.6.4 - Dominant lethal assay for the male fertility

In the two-generation study, no effects on reproductive parameters occurred. Therefore, this type of study is not required.KIIA 5.6.4 - Dominant lethal assay for the male fertility

In the two-generation study, no effects on reproductive parameters occurred. Therefore, this type of study is not required.

KIIA 5.6.5 - Cross-matings of treated males with untreated females and vice versa

In the two-generation study, no effects on reproductive parameters occurred. Therefore, this type of study is not required.

KIIA 5.6.6 - Effects on spermatogenesis

In the two-generation study, no effects on reproductive parameters occurred. Therefore, this type of study is not required.

KIIA 5.6.7 - Effects on oogenesis

In the two-generation study, no effects on reproductive parameters occurred. Therefore, this type of study is not required.

KIIA 5.6.8 - Sperm motility, mobility and morphology

In the two-generation study, sperm motility, mobility and morphology were assessed. Since there were no effects on sperm parameters, further studies were not required.

KIIA 5.6.9 - Investigation of hormonal activity

As no effects on the reproductive parameters were seen in the two-generation study, no investigations on the hormonal activity were required.

KIIA 5.6.10 - Teratogenicity test by the oral route in the rat

Report:	**KIIA 5.6.10/01, [redacted] C.; 2010**
Title:	BYI 02960 - Developmental toxicity study in the rat by gavage
Report No & Document No	SA 08347 M-363938-01-2
Guidelines:	OECD 414 (2001); EPA Health Effects Test Guideline (OPPTS 870.3700; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
GLP	Yes (certified laboratory)

Executive Summary

In a developmental toxicity study BYI 02960 (batch number 2009-000239, 96.2% w/w purity) was administered daily by gavage from gestation day (GD) 6 to 20 to groups of 25 pregnant Sprague-Dawley female rats per dose-group. The doses given were 0, 15, 50 and 150 mg/kg/day in suspension in aqueous solution of 0.5% methylcellulose 400.
Clinical observations were recorded daily and body weights were recorded for all females on GD 0, 6, 8, 10, 12, 14, 16, 18 and 21. Food consumption was also measured for all the females during the intervals GD 1-6, 6 - 8, 8 - 10, 10 - 12, 12 - 14, 14 - 16, 16 - 18 and 18 - 21. At scheduled sacrifice, on GD 21, a macroscopic examination of the visceral organs was performed, the gravid uterine weight was recorded and the dams were evaluated for number of corpora lutea, number and status of implantations (resorptions, dead and live fetuses). In addition, the liver was weighed at scheduled sacrifice for all pregnant females. A portion of liver was retained in 10% neutral buffered formalin from all females on study for possible histological examination. Specimens were not examined and are retained in the archives. Live fetuses were removed from the uteri, counted, weighed, sexed and examined externally. Approximately half of the live fetuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half were eviscerated, fixed in absolute ethanol and stained according to a modification of the Tyl and Marr technique for skeletal examination of bone and cartilage.

Pregnancy rate in all treated groups was similar to the controls with at least 96% pregnant females per group. No treatment-related mortalities occurred during the study. At necropsy, there were no treatment-related macroscopic findings in any dose group. At cesarean section, the number of live fetuses per litter, number of implant sites per dam, percentages of pre- and post-implantation losses, early or late resorptions, fetal death status and percentage of male fetuses were unaffected by treatment. There were no treatment-related fetal malformations or external or visceral variations.

<u>At 150 mg/kg/day</u>
Increased salivation was noted in 20/23 animals on one or several occasions between GD 11 and 21 and soiling around the mouth was observed in 1/23 females on GD 21. There was a mean maternal body weight loss of 5.7 g between GD 6-8, compared to a weight gain of 5.9 g in the concurrent controls. In addition, between GD 8-10, the mean body weight gain was reduced by 24% when compared to the control group. As a consequence, the mean maternal body weight changes were statistically

significantly lower than controls by 14 to 91% on all intervals between GD 6 and GD 18. The overall body weight gain between GD 6 - 21 and the maternal corrected body weight change (maternal body weight gain during gestation - gravid uterine weight) were reduced by 7 and 16%, respectively, compared to controls (not statistically significant). Mean food consumption was reduced by between 9 and 27% on all intervals between GD 6 and 12. Thereafter mean food consumption was comparable to controls. At necropsy, the mean absolute liver weight was 13% higher than controls.
At cesarean section, mean fetal body weights for combined sexes and females were reduced by 2 to 3% compared to the controls (not statistically significant). At the fetal skeletal examination, the incidences of two variations ("parietal (uni/bi): incomplete ossification" and "hyoid centrum: incomplete ossification") were higher than in the control group and were indicative of a slightly delayed fetal development.

At 50 mg/kg/day
The mean maternal body weight gain was reduced by 49% and mean food consumption was reduced by 8% between GD 6-8, when compared to the concurrent controls. As a consequence, the mean maternal body weight change was statistically significantly lower than controls by 23% between GD 6 and GD 10 ($p < 0.05$).
There were no treatment-related effects at the fetal evaluation.

At 15 mg/kg/day
There were no treatment-related effects.

In conclusion, a dose level of 150 mg/kg/day BYI 02960 administered to the pregnant female Sprague-Dawley rat caused maternal toxicity, as evidenced by clinical signs, strong body weight loss and reduced food intake, and slightly delayed fetal development, as evidenced by reduced fetal body weights and reduced ossification of a few skull bones. A dose level of 15 mg/kg/day was a No Observed Adverse Effect Level (NOAEL) for maternal toxicity based on a significant reduction in weight gain and food consumption in dams administered 50 mg/kg. A dose level of 50 mg/kg/day was a NOEL for developmental toxicity.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable in suspension in the vehicle (aqueous solution of methylcellulose 400 at 0.5%) at concentrations of 0.1 and 250 g/L for a period of 28 days under similar conditions to those of the current study

2. Vehicle and /or positive control: The vehicle was an aqueous solution of methylcellulose 400 at 0.5%

3. Test animals:

Species:	Rat
Strain:	Sprague Dawley Crl:CD(SD)
Age:	12 to 16 weeks approximately
Weight at study initiation (GD0):	225 to 289 g for the females
Source:	[redacted] France
Acclimation period:	12 days prior to mating
Diet:	Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 55 ± 15% Air changes: Approximately 10 to 15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates

February 18 to December 11, 2009 at [redacted], France.

2. Animal assignment and treatment

One hundred and twenty adult nulliparous female rats were obtained from the supplier. 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. The day where evidence of mating was found, was designated as GD 0. The females were assigned to control and treated groups each day of pairing using a body weight dependent randomization procedure. If possible, those females having been paired with the same male were not allocated to the same group. Body weight means were checked after the mating period to ensure similar means among all groups.

3. Dose selection rational

The range of doses was selected based on results obtained in a range-finding study where gavage administration to 8 pregnant rats per group at 50, 100, 200 and 350 mg/kg bw/day from GD 6 to 20 resulted in maternal toxicity at 350 and 200 mg/kg/day, as evidenced by clinical signs (increased salivation, soiled mouth, soiled anogenital region or vaginal discharge), reduced body weight gain and corrected body weight change, reduced food consumption and increased liver weight at necropsy. In addition, the percentages of pre and post implantation losses were increased at 350 mg/kg/day, and the

number of live fetuses per litter was decreased at both dose levels. Mean fetal body weight was reduced at both dose levels, and a few variations were noted at the external and skeletal observation at 350 mg/kg/day only. A dose level of 100 mg/kg/day induced slight maternal toxicity (reduced body weight gain and food consumption between GD 6 - 8) and slight developmental toxicity (lower number of live fetuses per litter and lower mean fetal body weight). A dose level of 50 mg/kg/day did not induce treatment-related changes in the parameters assayed.

4. Test substance dosage formulations and analysis

The appropriate amount of BYI 02960 was suspended (w/v) in an aqueous solution of methylcellulose 400 (Fluka, Mulhouse, France) at 0.5% and stored at approximately 5 °C (± 3 °C). Formulations were prepared twice (F1 and F2) during the study. Homogeneity of the suspensions was checked on the first formulation (F1) for the lowest and the highest concentrations 1.5 and 15 g/L). In addition, the intermediate concentration of the first formulation (F1) and all concentrations of the second formulation (F2) were checked. Homogeneity and concentration checks were between 93 and 101% of nominal values. Stability of the test substance in suspension in the vehicle at concentrations of 1 and 250 g/L was determined in a previous study and was found to be stable for 28 days under similar conditions to those of the current study.

Table 5.6.10-01: Study design and animal assignment

Test group	Test substance	Dose levels mg/kg/day	Concentrations g/L	Volume (mL/kg)	Number of animals
1	0	0	0	10	23
2	BYI 02960	15	1.5	10	23
3		50	5.0	10	23
4		150	15.0	10	23

Doses were administered daily by gavage to each female from GD 6 to 20, based on the animal's most recent body weight, and at a volume of 10 mL/kg. Control animals received an equivalent volume of vehicle alone (0.5% aqueous methylcellulose).

C. Methods

1. Observations

All rats were observed daily for clinical signs and twice daily for mortality (except at weekends and public holidays when checking was carried out once daily).

2. Body weight

Body weights were measured on GD: 0, 6, 8, 10, 12, 14, 16, 18 and 21.

3. Food consumption

Food consumption was measured at the following intervals: full feeder on GD: 1, 6, 8, 10, 12, 14, 16 and 18 and empty feeder weights were measured on GD: 6, 8, 10, 12, 14, 16, 18, and 21.

4. Caesarian sections

On GD 21, all females were sacrificed by inhalation of carbon dioxide, for examination of uterine content. Autopsies were performed blind with regard to the animal study identification. Each female was first subjected to macroscopic examination of the visceral including examination of the liver. The liver of all pregnant females was weighed. The liver from all females on study was retained in 10% neutral buffered formalin for possible histological examination. Liver samples were not examined and are retained in the archives.
The reproductive tract was weighed (gravid uterine weight), dissected out and the following parameters recorded: number of corpora lutea, number of implantation sites, number of resorption sites (classified as early and late), number of live and dead fœtuses, sex and individual weights of live fœtuses. Dead fœtuses: were defined as fœtuses showings distinct digits visible on fore and hind-paws. Runt fœtuses were defined as live fœtuses weighing less than 4 g at Caesarian section of the dam. Uterine horn(s) without visible implantations were immersed in a 10% solution of ammonium sulfide to visualize any sites which were not apparent. Intra-uterine death was classified as early resorptions when macroscopic discrimination between fœtal residues and placental material was not possible and late resorption when distinct macroscopic discrimination between fœtal residues and placental material was possible.

5. Foetal examination
All data were recorded without knowledge of treatment group. All live fœtuses were subjected to external examination and then sacrificed by subcutaneous injection (0.02 mL/foetus) of Dolethal (18.22 g/100 mL, sodium pentobarbital). Approximately half of the live fœtuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half were eviscerated, fixed in absolute ethanol and stained according to a modification of the Tyl and Marr technique for skeletal examination of bone and cartilage.
Structural deviations were classified as malformations, minor anomalies, or common variants according to Palmer (1977).
6. Statistics
Means and standard deviations (STD) for all maternal and litter parameters were calculated for each group.
Maternal endpoints
Statistical analyses, as described below, were performed on the following parameters using Teratest Phase 1, Version 12:

- Body weight changes calculated according to interval periods
- Calculated corrected body weight change
- Average food consumption calculated according to interval periods
- Liver weight.

Body weight data measured on different days throughout gestation were not statistically analyzed; only descriptive statistics are presented.

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant.

Litter based and fœtal endpoints

Statistical analyses, as described below, were performed on the following parameters using Teratest Phase 2, Version 4:

- Number of corpora lutea
- Number of implantation sites
- Number of resorptions (early, late)
- Pre- and post-implantation loss percentages.

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance.

Statistical analyses, as described below, were performed on the following parameter using SAS programs (Version 8.2):

- Fetal body weight (combined sexes and per sex).

For fetal body weight (combined sexes and per sex) endpoint, statistical analyses were performed using the mean fetal body weight per litter as the statistical unit.

Data were analyzed by the Levene's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant.

Statistical analyses, as described below, were performed on the following parameters using Teratest Phase 2, Version 4:

- Fetal sex (male *vs.* female described in terms of percent male fetuses)
- Fetal death status (live *vs.* dead, described in terms of number of dead fetuses and number of litters with dead fetuses).

For fetal sex (male *vs.* female fetuses) and fetal death status (live *vs.* dead fetuses) endpoints, control group and each exposed group were compared using the Chi-square test for fetal sex parameter, using the Fisher Exact test (2-sided) for fetal death status parameter. Death status was analyzed both using the fetus as the statistical unit and using the litter as the statistical unit.

Group means were compared at the 5% and 1% levels of significance.

In addition, a statistical analysis was performed on selected fetal skeletal observation data.

II. Results and discussion

A. Maternal observations

1. Mortality

There were no mortalities during the course of the study.

2. Clinical signs

At 150 mg/kg/day, increased salivation was noted in 20/23 animals on one or several occasions between GD 11 and 21 and soiling around the mouth was observed in 1/23 females on GD21.
There were no treatment-related clinical signs at 50 and 15 mg/kg/day.

3. Pregnancy rate

The pregnancy rate was at least 96% in all dose groups, comparable to control.

4. Body weight

There were no statistically significant changes in mean body weights at any dose level.
At 150 mg/kg/day, there was a mean maternal body weight loss of 5.7 g between GD 6 - 8, compared to a weight gain of 5.9 g in the concurrent control. In addition, between GD 8-10, the mean body weight gain was reduced by 24% when compared to the control group. As a consequence, the mean maternal body weight changes were statistically significantly lower than controls by 14 to 91% on all intervals between GD 6 and GD 18. The overall body weight gain between GD 6 - 21 and the maternal corrected body weight change (maternal body weight gain during gestation - gravid uterine weight) were reduced by 7 and 16%, respectively, compared to controls (not statistically significant).

At 50 mg/kg/day, the mean maternal body weight gain was reduced by 49% between GD 6 - 8, compared to the control group (not statistically significant). As a consequence, the mean maternal body weight change was statistically significantly lower than controls by 23% between GD 6 and GD 10. Thereafter the mean body weight gain was comparable to controls and there was no effect on the maternal corrected body weight change.

At 15 mg/kg/day, there were no treatment-related changes in mean body weight changes and corrected body weight change. In the absence of dose dependency and as no effect was noted on interval GD 6 -8, the apparent reduction in mean body weight gain observed on interval GD 8-10 was considered not to be treatment-related.

Table 5.6.10-02: Summary table on maternal body weight gain (g)

Intervals	BYI 02960 dose levels in mg/kg/day				HCD[#]
	0	15	50	150	
No. of dams (pregnant)	22	22	22	23	
Pretreatment GD 0 - 6	29.4 ± 8.08	25.3 ± 7.50	27.6 ± 5.53	28.5 ± 5.44	28.05 - 39.25
Treatment GD 6 - 8	5.9 ± 3.82	5.9 ± 2.78	3.0 ± 3.75	- 5.7 ± 5.18 **	4.05 - 8.48
Treatment GD 8 - 10	9.1 ± 1.96	7.4 ± 2.8*	8.5 ± 2.56	6.9 ± 3.48*	7.78 - 11.30

Treatment GD 10 - 14	16.8 ± 3.84	17.1 ± 7.81	16.1 ± 5.22	19.5 ± 4.09	17.17 - 22.33
Treatment GD 14 - 18	41.0 ± 8.88	43.8 ± 7.06	44.9 ± 6.48	41.7 ± 7.64	41.48 - 48.21
Treatment GD 18 - 21	43.6 ± 9.61	44.9 ± 17.09	47.5 ± 7.59	45.7 ± 8.89	47.05 - 58.21
Treatment GD 6 - 21	116.4 ± 18.77	119.1 ± 25.52	120.1 ± 16.94	108.2 ± 16.75	122.6 - 143.3
Corrected BW gain	44.6 ± 16.80	40.9 ± 18.89	44.0 ± 12.81	37.3 ± 8.89	49.56 - 75.43

HCD : Historical control range (lowest - highest) from Att.3 in the Tables and Appendices section of the study report
** : p <0.01 * : p <0.05

5. Food consumption

At 150 mg/kg/day, mean food consumption was reduced by between 9 and 27% on all intervals between GD 6 and 12. Thereafter mean food consumption was comparable to controls.
At 50 mg/kg/day, mean food consumption was slightly reduced by 8% between GD 6 - 8, when compared to the concurrent controls.
At 15 mg/kg/day, mean food consumption was comparable to the controls.

6. Maternal necropsies and microscopic findings

At necropsy, the mean absolute liver weight was 13% higher than controls at 150 mg/kg/day.
There were no relevant changes in absolute liver weights at 50 or 15 mg/kg/day.
There were no treatment-related findings at the macroscopic examination at any dose level.

B. Litter data

The number of live fetuses, the number of implant sites per dam, the percentages of pre and post implantation losses, the number of early and late resorptions, the fetal death status and the percentage of male fetuses were unaffected by treatment in any dose group.
At 150 mg/kg/day, mean fetal body weights for combined sexes and females were reduced by 2 to 3%, compared to the controls (not statistically significant).
There were no treatment-related effects on mean fetal body weights at 50 or 15 mg/kg/day. The decrease in mean fetal body weights observed at 15 mg/kg/day was considered not to be treatment related, as it was due to one outlier female, which had a severe body weight loss between GD 10 - 14 and between GD 18 - 21 and a strong reduction in food intake on all intervals between GD 10 and 21. This female had also a very high liver weight at necropsy.

Table 5.6.10-03: Cesarean section observations

Observation	Dose Level of BYI 02960 (mg/kg/day)				HCD
	0	15	50	150	
Maternal data:					
No. Animals assigned	23	23	23	23	NA
No. Animals pregnant	22	22	22	23	NA
Pregnancy rate, %	96	96	96	100	NA
No. Animals non-pregnant	1	1	1	0	NA
Maternal wastage					
No. died (total)	0	0	0	0	NA
No. died pregnant	0	0	0	0	NA
No. died non-pregnant	0	0	0	0	NA
No. premature delivery	0	0	0	0	NA
Uterine data:					
Total No. corpora lutea[c]	370	380	361	377	NA
Corpora lutea / dam	16.8 ± 2.0	17.3 ± 2.4	16.4 ± 1.7	16.4 ± 2.2	15.74 - 17.83
Total No. implantations[c]	329	349	334	345	NA
Implantations / dam	15.0 ± 2.8	15.9 ± 1.8	15.2 ± 1.8	15.0 ± 2.4	14.38 - 16.04
Total No. litters[c]	22	22	22	23	
Total No. live fetuses[c]	304	319	313	314	NA
Live fetuses / dam[c]	13.8 ± 3.0	14.5 ± 2.2	14.2 ± 2.3	13.7 ± 2.5	13.52 - 15.04
Total No. dead fetuses	0	0	0	0	NA
Dead fetuses / dam[c], %	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 - 0.30
Total No. early resorptions[c]	21	28	21	30	NA
Total No. late resorptions[c]	4	2	0	1	NA
Early resorptions / dam	1.0 ± 1.1	1.3 ± 1.3	1.0 ± 1.3	1.3 ± 1.3	0.474 - 2.042
Late resorptions / dam	0.2 ± 0.4	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.2	0.0 - 0.217
Litters with total resorptions[c]	0	0	0	0	NA
Mean fetal weight, combined sexes, g	5.41 ± 0.28	5.27 ± 0.60	5.35 ± 0.24	5.27 ± 0.25	5.30 - 5.57
Mean fetal weight, males, g	5.50 ± 0.28	5.36 ± 0.60	5.46 ± 0.29	5.40 ± 0.29	5.45 - 5.70
Mean fetal weight, females, g	5.26 ± 0.29	5.18 ± 0.59	5.23 ± 0.24	5.14 ± 0.25	5.16 - 5.42
Sex ratio, % male	51.3 ± 12.3	52.9 ± 14.1	46.7 ± 15.4	51.7 ± 10.3	46.2 - 53.5
Preimplantation loss per litter, %	10.5 ± 16.2	7.4 ± 9.2	7.2 ± 9.3	8.3 ± 10.6	3.32 - 13.21
Postimplantation loss per litter, %	7.9 ± 10.3	8.6 ± 8.4	6.5 ± 9.3	9.0 ± 8.3	3.95 - 13.15

* : Statistically different ($p \leq 0.05$) from the control
** : Statistically different ($p \leq 0.01$) from the control
[c] : Statistical analysis was not conducted on this endpoint
HCD : Historical control range (lowest - highest) of main uterine parameters
NA : not applicable

C. Fœtal necropsy findings

1. Fœtal evaluation: external observations

There were no treatment-related malformations or variations noted at the fetal external examination.
Two fetuses in two different litters were observed with malformations in the low dose group: one was omphalocele and one had an absence of eye bulge (unilateral). In the absence of dose dependency, they were considered to have occurred spontaneously. The two other variations observed occurred as isolated findings and were considered incidental.

2. Fœtal evaluation: visceral observations

There were no treatment-related malformations or variations noted at the fetal visceral examination.
One fetus from the low dose group and one fetus from the high dose group were observed with malformations: one had unilateral microphtalmia (corresponding to the eye bulge absent observed at the external fetal examination) and one had a severe dilatation of renal pelvis and dilated and convoluted ureters (bilateral). In isolation and since the incidences of eye bulge absent and severe dilatation of renal pelvis and dilated and convoluted ureters are within the in-house HCD, these malformations were considered to have occurred spontaneously.
The others variations observed did not occur in a dose-related manner and were well in the in-house HCD or occurred as isolated findings and were considered incidental.

3. Fœtal evaluation: skeletal observations

There were no treatment-related malformations noted at the fetal skeletal examination.
One fetus from the control group was observed with thoracic malformations.
At 150 mg/kg/day, the incidence of the variations "parietal (uni/bi): incomplete ossification" and "hyoid centrum: incomplete ossification" were higher than in the control group and were outside the in-house HCD at both litter and fetal levels. These findings were indicative of a slightly delayed fetal development and considered to be treatment-related.

There were no other treatment-related fetal skeletal variations observed at any dose level.
The incidence of the variations "7th cervical centrum: unossified" and "5th sternebra: incomplete ossification" were higher at 150 mg/kg/day than in the control group. The differences were not statistically significant. In the absence of a clear dose dependency and as all values were well within the in-house HCD at both the litter and fetal level, these findings were considered not to be treatment-related.
The incidence of "13th costal cartilage (uni): short" was higher at 150 mg/kg/day than in the control group and was outside the in-house HCD at both litter and fetal levels. However, as the observation was noted unilaterally only, the incidence was low (2 cases in 2 litters) and the finding was of minor biological relevance, this increased incidence was considered not to be treatment-related.
The other variations observed did not occur in a dose-related manner and were well within the in-house HCD or occurred as isolated findings and were considered incidental.

Table 5.6.10-04: Skeletal examinations

Dose level of BYI 02960 (mg/kg/day)	0	15	50	150	Historical Control Range	0	15	50	150	Historical Control Range
Observations	Number of litters examined					Number of fetuses examined				
	22	22	22	23		158	165	161	162	
	Number of litters affected (% of litters affected)					Number of fetuses affected (% of fetuses affected)				
Variations # Parietal (uni/bi) : incomplete ossification.	0 (0.0)	2 (9.1)	0 (0.0)	4 (17.4)	(0.0 9.1)	0 (0.0)	6 (3.6)*	0 (0.0)	9 (5.6)**	(0.0 1.3)
# Hyoid centrum : incomplete ossification.	0 (0.0)	2 (9.1)	1 (4.5)	4 (17.4)	(0.0 12.5)	0 (0.0)	2 (1.2)	1 (0.6)	9 (5.6)**	(0.0 1.9)
# 7th cervical centrum : unossified.	2 (9.1)	2 (9.1)	4 (18.2)	7 (30.4)	(0.0 41.7)	6 (3.8)	7 (4.2)	4 (2.5)	11 (6.8)	(0.0 12.2)
# 5th sternebra: incomplete ossification.	10 (45.5)	11 (50.0)	7 (31.8)	12 (52.2)	(19.0 70.8)	17 (10.8)	21 (12.7)	8 (5.0)*	24 (14.8)	(3.1 19.9)
13th costal cartilage (uni) : short.	0 (0.0)	0 (0.0)	0 (0.0)	2 (8.7)	(0.0 0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.2)	(0.0 0.0)

: Statistical analysis was conducted on this observation
* : Statistically different ($p \leq 0.05$) from the control
** : Statistically different ($p \leq 0.01$) from the control

III. Conclusions

In conclusion, a dose level of 150 mg/kg/day BYI 02960 administered to the pregnant female Sprague-Dawley rat caused maternal toxicity, as evidenced by clinical signs, strong body weight loss and reduced food intake, and slightly delayed fetal development, as evidenced by reduced fetal body weights and reduced ossification of a few skull bones.
A dose level of 15 was the NOAEL for maternal toxicity and a dose level of 50 mg/kg/day was a NOAEL for developmental toxicity.

Report:	**KIIA 5.6.10/02, [redacted] C.; 2012**
Title:	BYI 02960 - Developmental toxicity study in the rat by gavage
Report No & Document No	SA 11140 M-425810-01-2
Guidelines:	Not applicable complementary study on maternal toxicity
GLP	Yes (certified laboratory)

Executive Summary

This study was designed to provide additional information on the maternal tolerability of BYI 02960 (batch number: 2009-000239, a beige powder, 96.2% w/w purity) and determine more precisely the No Observed Effect Level (NOEL) for maternal toxicity.
Groups of 23 sperm-positive female Sprague-Dawley rats were exposed to BYI 02960 by oral gavage from gestation day (GD) 6 to 20; the sperm-positive day being GD 0. The doses given were 0, 20 and 30 mg/kg body weight/day in suspension in aqueous solution of 0.5% methylcellulose 400. The volume of administration was 10 mL/kg based on the most recent body weight recorded.

Clinical observations were recorded daily. Maternal body weights were recorded for all females on GD 0, 6, 8, 10, 12, 14, 16, 18 and 21. Food consumption was also measured for all the females during the intervals GD 1 - 6, 6 - 8, 8 - 10, 10 - 12, 12 - 14, 14 - 16, 16 - 18 and 18 - 21. At scheduled sacrifice, on GD 21, a macroscopic examination of the visceral organs was performed, the gravid uterine weight was recorded and the live fetuses were euthanized. In addition, the liver was weighed at scheduled sacrifice for all pregnant females. A portion of liver was retained in 10% neutral buffered formalin from all females on study for possible histological examination. Specimens were not examined and are retained in the archives.

Up the highest dose level tested of 30 mg/kg/day, there were no treatment-related maternal effects.

The dose level of 30 mg/kg of BYI 02960 /day is considered to be the No Observed Adverse Effect Level (NOAEL) for maternal toxicity.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960

Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2 %
CAS:	951659-40-8
Stability of test compound:	Stable in suspension in the vehicle (aqueous solution of methylcellulose 400 at 0.5%) at concentrations of 0.1 and 250 g/L for a period of 28 days under similar conditions to those of the current study

2. Vehicle and /or positive control: The vehicle was an aqueous solution of methylcellulose 400 at 0.5%

3. Test animals:

Species:	Rat
Strain:	Sprague Dawley Crl:CD(SD)
Age:	12 to 15 weeks approximately
Weight at study initiation (GD0):	230 to 278 g for the females
Source:	[redacted] France
Acclimation period:	At least 12 days prior to mating
Diet:	Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 55 ± 15% Air changes: Approximately 10 to 15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design and methods

1. In life dates

October 26 to December 01, 2011 at [redacted], France.

2. Animal assignment and treatment

One hundred and twenty adult nulliparous female rats were obtained from the supplier. Females were mated on a one-to-one basis with stock males of the same strain and same supplier. Each morning following pairing, those female rats showing spermatozoa in a vaginal smear or sperm plug *in situ* were considered as pregnant animals. The day where evidence of mating was found, was designated as gestation Day 0 (GD 0). The females were assigned to control and treated groups using a body weight procedure for each day of pairing. If possible, those females having been paired with the same male were not allocated to the same group. Body weight means were checked after the mating period to ensure similar means among all groups. Permanent identification numbers were assigned to animals within each group. Each animal was identified by a cage card and an ear tag bearing a unique number. The dose groups are indicated in the following table:

Table 5.6.10-05: Study design

Test Item	Dose levels (mg/kg/day)	Concentrations g/L	Volume mL/kg	Number of animals
Vehicle	0	0	10	23
BYI 02960	20	2	10	23
	30	3	10	23

All doses were administered once daily by oral gavage, on GD 6 to 20 inclusive in a volume of 10 mL/kg body weight/day. Dosing was based on the animal's most recent recorded body weight. Control animals received an equivalent volume of vehicle alone.

3. Dose selection rational

The range of doses were selected based on results obtained in the developmental toxicity study in the rat presented above where pregnant rats received 0, 15, 50 and 150 mg/kg body weight/day BYI 02960 from GD 6 to 20. In this study, the dose level of 15 mg/kg/day was the No Observed Effect Level (NOEL) for maternal toxicity, based on effects observed at 50 mg/kg body weight/day; the mean maternal body weight gain was reduced by 49% and mean food consumption was reduced by 8% between GD 6 - 8, when compared to the concurrent controls. There were no treatment-related effects at the cesarean or fetal evaluation at 50 or 15 mg/kg/day.

4. Test substance dosage formulations and analysis

The appropriate amount of test item was suspended (w/w) in an aqueous solution of methylcellulose 400 at 0.5% and stored in air-tight bottles at approximately 5°C (± 3 °C). Test formulations were prepared twice (F1 and F2) during the study.
Stability of the test item in 0.5% aqueous methylcellulose 400 was demonstrated in study SA 07025 at concentrations of 1 and 250 g/L for up to 28 days under similar conditions of usage and storage to those of the current study. Homogeneity of the suspensions was checked on the first formulation (F1) at all concentrations (2 and 3 g/L). The mean values obtained from the homogeneity check were used as measured concentrations. In addition, all concentrations of the second formulation (F2) were checked. All homogeneity and concentration analyses were within 96 to 99% of nominal concentrations, which is within the in-house target range of 90 to 110% of nominal concentration. Therefore dose preparations were considered acceptable for use on this study.

5. Statistics

Means and standard deviations (STD) for all maternal parameters were calculated for each group.
Maternal endpoints
Statistical analyses, as described below, were performed on the following parameters using Path/Tox System V4.2.2. (Module Enhanced Statistics):

- Body weight changes calculated according to interval periods
- Calculated corrected body weight change
- Average food consumption calculated according to interval periods
- Liver weight.

Body weight data measured on different days throughout gestation were not statistically analyzed; only descriptive statistics are presented.

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant.
If one or more group variance(s) equaled 0, means were compared using non-parametric procedures.
Group means were compared at the 5% and 1% levels of significance.

C. Methods

1. Observations
Clinical signs
All clinical signs were recorded for individual animals. All animals were examined daily from GD 0 through GD 21.

Mortality
All cages were checked for dead or moribund animals twice daily, once in the morning and again in the afternoon (except at weekends and public holidays when checking was carried out once daily).

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

3. Food consumption
Full feeder weights were measured on GD: 1, 6, 8, 10, 12, 14, 16 and 18.
Empty feeder weights were measured on GD: 6, 8, 10, 12, 14, 16, 18 and 21.
From these records the mean daily consumption was calculated. Food spillage was also noted.

4. Cesarian sections
Animals found dead or killed *in extremis*
Animals found dead were subjected to a macroscopic examination of the visceral organs. The liver was retained in 10% neutral buffered formalin from all females for possible histological examination. The pregnancy status was determined. In the case of no visible uterine implants, uterine horn(s) were immersed in a 10% solution of ammonium sulfide according to the Salewski method, in order to visualize any sites which were not apparent. Then, tissues and carcass of dams were discarded.

Scheduled sacrifice
On GD 21, all surviving females were killed by inhalation of carbon dioxide. Each female was first subjected to macroscopic examination of the visceral organs. The liver of all pregnant females was weighed and retained in 10% neutral buffered formalin from all females for possible histological examination. Liver samples were not examined and are retained in the archives.
The pregnancy status was determined and the reproductive tract was weighed (gravid uterine weight). Uterine horn(s) without visible implantations were immersed in a 10% solution of ammonium sulfide

according to the SALEWSKI method in order to visualize any sites which were not apparent. Then, tissues and carcass of dams were discarded.

5. Fetal examination

All the live fetuses were killed by intrathoracic injection (0.02 mL/fetus) of Dolethal® (18.22 g/100 mL, sodium pentobarbital) and discarded without examination.

II. Results and discussion

A. Maternal observations

1. Mortality

There was no treatment-related mortality.

One animal, treated at 20 mg/kg/day, died on GD 20 after having been dosed. Nasal discharge and soiling around the mouth were noted at the clinical examination after dosing. Necropsy revealed red dark lungs and presence of foam in lungs and trachea, indicative of a gavage error.

2. Clinical signs

There were no treatment-related clinical signs.

Hunched posture and piloerection were observed for 2 days in 1/23 animals at 20 mg/kg/day. In isolation and in the absence of dose-relationship, these signs were considered not to be related to the treatment.

3. Body weights

Body weight parameters were unaffected by treatment. Body weight gain data are summarized in the following table.

Table 5.6.10-06: Mean (± SD) maternal body weight gain (g)

Interval	Dose level of BYI 02960 in mg/kg/day		
	0	20	30
Number of dams (pregnant)	21	23	23
Pretreatment, GD 0-6:	29 ± 5	28 ± 6	30 ± 8
Treatment, GD 6-8:	4 ± 4	4 ± 4	3 ± 4
Treatment, GD 8-10:	10 ± 4	10 ± 4	9 ± 4
Treatment, GD 10-14:	20 ± 5	19 ± 5	21 ± 5
Treatment, GD 14-18:	46 ± 9	46 ± 8	47 ± 8
Treatment, GD 18-21:	53 ± 9	52 ± 9	53 ± 10
Treatment, GD 6-21	133 ± 19	131 ± 18	133 ± 20
Corrected BW gain	55.6 ± 12.0	53.9 ± 8.5	58.0 ± 14.2

4. Food consumption

Food consumption was unaffected by treatment.

5. Gross pathology and organ weights

There were no treatment-related macroscopic findings at necropsy and no changes in liver weights at any dose level. Cesarean section data are summarized in the following table.

Table 5.6.10-07: Cesarean section observations

Observation	Dose Level of BYI 02960 (mg/kg/day)		
	0	20	30
Maternal data:			
No. Animals assigned	23	23	23
No. Animals pregnant	21	23	23
Pregnancy rate, %	91	100	100
No. Animals non-pregnant	2	0	0
Maternal wastage			
No. died (total)	0	1	0
No. died pregnant	0	1	0
No. died non-pregnant	0	0	0
No. premature delivery	0	0	0

III. Conclusions

Up the highest dose level tested of 30 mg/kg/day, there were no treatment-related maternal effects. The dose level of 30 mg/kg of BYI 02960 /day is considered to be the No Observed Adverse Effect Level (NOAEL) for maternal toxicity.

KIIA 5.6.11 - Teratogenicity test by the oral route in the rabbit

Report:	**KIIA 5.6.11/01, [redacted]; 2012**
Title:	BYI 02960 - Developmental toxicity study in the rabbit by gavage
Report No & Document No	SA 10314 M-423559-02-1
Guidelines:	OECD 414 (2001); EPA Health Effects Test Guideline (OPPTS 870.3700; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
GLP	Yes (certified laboratory)

Executive Summary

Groups of 23 time-mated pregnant female New Zealand White rabbits were exposed to BYI 02960 (batch number 2009-000239, a beige powder, 96.2% w/w purity), by oral gavage from gestation day (GD) 6 to 28, the day of mating being GD 0. The doses given were 0, 7.5, 15 and 40 mg/kg body weight/day in suspension in aqueous solution of 0.5% methylcellulose 400. The volume of administration was 4 mL/kg based on the most recent body weight recorded.

Clinical observations were recorded daily. Maternal body weights were recorded for all females on GD 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Food consumption was also measured for all the females during the intervals GD 3 - 4, 4 - 5, 5 - 6, 6 - 8, 8 - 10, 10 - 12, 12 - 14, 14 - 16, 16 - 18, 18 -20, 20 - 22, 22 - 24, 24 - 26, 26 - 28 and 28 - 29. At scheduled sacrifice, on GD 29, a macroscopic examination was performed, the gravid uterine weight was recorded and the dams were evaluated for

number of corpora lutea, number and status of implantations (resorptions, dead and live fetuses). In addition, the liver was weighed at scheduled sacrifice from all pregnant females. A portion of the liver was retained in 10% neutral buffered formalin from all females for possible histological examination. Live fetuses were removed from the uteri, counted, weighed and examined externally. The heads of fetuses from approximately half of each litter were immersed in Bouin's fluid and the internal structures examined after fixation. The bodies of all fetuses were dissected for soft tissue anomalies and sex determination. Fetuses were eviscerated, skinned and fixed in absolute ethanol before staining. A modification of the Staples and Schnell technique was applied and a subsequent skeletal examination was performed.

Pregnancy rate was unaffected by treatment, with a pregnancy rate of 96% at 7.5 and 40 mg/kg/day and 100% in the control group and at 15 mg/kg/day.

At 40 mg/kg/day

In dams, no treatment-related clinical signs were observed. There was a mean body weight loss of 0.01 g in all pregnant females between GD 6 and 8 compared to a mean body weight gain of 0.02 g in control animals; this effect was considered to be treatment-related, though it was not statistically significant and remained within the range of in-house Historical Control Data (HCD), since mean maternal body weight between GD 6 and 10 was lower than the controls (-67%, $p \leq 0.05$). Thereafter, mean body weight gain was comparable to the controls throughout all intervals. Mean maternal body weights were comparable to the controls whilst mean maternal corrected body weight change (maternal body weight change between GD 6 and 29 - gravid uterine weight) was 12% lower than the controls (not statistically significant) and slightly outside the range of in-house HCD. Mean maternal food consumption was reduced by 20% between GD 6 to 8 ($p \leq 0.01$) and by 11% between GD 8 to 10 (not statistically significant). Thereafter, mean maternal food consumption was similar to the controls. At necropsy, no treatment-related macroscopic findings were noted and mean liver weight was similar to the controls.

At cesarean section, no treatment-related changes were noted on litter parameters, including the number of live fetuses, the number of implant sites per dam, the percentages of pre and post implantation losses, the number of early and late resorptions, the fetal death status, the fetal body weight and the percentage of male fetuses.

There were no treatment-related effects on litter parameters.

There were no treatment-related external, visceral or skeletal findings at the fetal examination.

At 15 and 7.5 mg/kg/day

No treatment-related maternal findings were noted.

There were no treatment-related effects on litter parameters.

There were no treatment-related external, visceral or skeletal findings at the fetal examination.

In conclusion, a dose level of 40 mg/kg/day BYI 02960 administered to the pregnant New Zealand White rabbit by oral gavage resulted in maternal toxicity as evidenced by body weight loss, significantly reduced body weight gain and food consumption between GD 6 and 10, and lower mean maternal corrected body weight change in compared to control animals. Fetal development was unaffected by treatment at any dose level tested. A dose level of 15 mg/kg/day was considered to be a No Observed Adverse Effect Level (NOAEL) for maternal toxicity, while a dose level of 40 mg/kg day was considered to be a NOAEL for developmental toxicity.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2 % w/w
CAS Number of TGAI:	951659-40-8
Stability of test compound:	Stable at room temperature (25 ± 5 °C) until 14 January 2013 (results from reanalysis performed on 14 January 2011)
2. Vehicle and/or positive control:	0.5% aqueous methylcellulose 400
3. Test animals:	
Species:	Rabbit (time-mated females)
Strain:	New Zealand White Crl:KBL (NZW)
Age:	18 ± 1 weeks old
Weight at GD3:	3.04 to 3.86 kg (first weighed at testing facility)
Source:	[redacted], France, animals were received on GD1 - 2
Acclimation period:	4 - 5 days prior to dosing
Diet:	Laboratory animal pellets 110C-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Animals were caged individually in polycarbonate cages on a perforated cage floor
Environmental conditions:	Temperature: 19 ± 2 °C Humidity: 55 ± 15% Air changes: 10 - 15 changes per hour Photoperiod: Alternating 16-hour light and 8-hour dark cycles (5 am - 9 pm)

B. Procedures and study design

1. In-life dates

3 January 2011 to 18 February 2011 at [redacted], France.

2. Animal assignment and treatment

106 time-mated female New Zealand White Crl:KBL (NZW) rabbits received on GD1-2 were used in this study. Stock males from the same strain were used by the supplier to naturally mate nulliparous females. The day of insemination was designated as GD 0. The animals were approximately 18 weeks of age on arrival and were received on GD 1 or 2. On each day of mating, the females were allocated to control and treated groups using a computerized randomization procedure (XMS Path/Tox Version 4.2.2). Body weight means were checked to ensure similar means among all groups. Permanent identification numbers were assigned to animals within each group. The doses were administered daily by gavage at a volume of 4 mL/kg to each female from GD 6 to GD 28 inclusive, based on the animal's most recent body weight. Control animals received an equivalent volume of vehicle alone (methylcellulose).

The experimental groups were as follows:

Table 5.6.11-01: Study design and animal assignment

Test group	Test substance	Dose levels mg/kg/day	Concentrations g/L	Volume (mL/kg)	Number of animals
1	Vehicle	0	0	4	23
2	BYI 02960	7.5	1.875	4	23
3		15	3.750	4	23
4		40	10.000	4	23

3. Dose selection rationale

The range of doses was selected based on results obtained in a range-finding study, where pregnant rabbits were administered the test material by gavage at 0, 15, 40, and 80 mg/kg/day from GD 6 to 28. A dose level of 80 mg/kg/day caused marked maternal toxicity as evidenced by clinical signs, body weight loss, and reduced food consumption. There were also an increased number and percentage of dead fetuses and a reduction in mean fetal body weight compared to controls at the cesarean section. No treatment-related changes were noted at the external fetal examination. At 40 mg/kg/day, there was slight maternal toxicity in the form of a mean body weight loss between GD 6 to 8, but no effects were observed on developmental parameters. At 15 mg/kg/day, there were no treatment-related effects on maternal or developmental parameters.

4. Test substance dosage formulations and analysis

The appropriate amount of test material was suspended (w/v) in an aqueous solution of 0.5% methylcellulose 400 (Fluka, Mulhouse, France) and stored at approximately 5 °C (± 3 °C). Test formulations were prepared six times (F1 to F6) during the study. Homogeneity of the suspensions was checked during the first formulation for the lowest (1.875 g/L) and highest (10.000 g/L) concentrations. The mean values obtained from the homogeneity check were used as measured concentrations. In addition the intermediate concentration of the first formulation (F1) and all concentrations of the remaining formulations (F2 to F6) were checked. Homogeneity and concentration checks were between 90 and 103% of nominal values, within the in-house target range of 90 to 110% of nominal concentration.

5. Dosage administration
All doses were administered once daily by oral gavage, on GD 6 to 28 inclusive in a volume of 4 mL/kg body weight/day. Dosing was based on the animal's most recent recorded body weight. Control animals received an equivalent volume of vehicle alone (aqueous 0.5% methylcellulose 400).

C. Method

1. Observations
All rabbits were observed daily for clinical signs and twice daily for mortality (except once daily on weekends and public holidays).

2. Body weight
Body weights were measured on GD: 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29.

3. Food consumption
Full feeder weights were measured on GD: 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28. Empty feeder weights were measured on GD: 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 29.

4. Caesarian sections
Animals killed *in extremis* or for humane reasons by intravenous injection of Dolethal® (18.22 g/100mL sodium pentabarbitol; Supplier: [redacted], France) (including females which aborted) or found dead, were subjected to a macroscopic examination of the visceral organs and were autopsied. The number and type of implantations and corpora lutea were noted when present. In the case of no visible uterine implants, but with corpora lutea, uterine horn(s) were immersed in a 20% solution of ammonium sulfide according to the Salewski method (1964) in order to visualize any sites which were not apparent. Tissues and carcasses of dams were then discarded.

On GD 29, surviving females were killed by intravenous injection of Dolethal® for examination of their uterine content. Each female was first subjected to macroscopic examination of the visceral organs and the number of ribs was recorded. In the case of no visible uterine implants, but with corpora lutea, uterine horn(s) were immersed in a 20% solution of ammonium sulfide according to the Salewski method. The liver of all pregnant females was taken and preserved in 10% neutral buffered formalin for possible histological examination. Tissues and carcasses were then discarded.

The reproductive tract was weighed (gravid uterine weight), dissected out and the following parameters recorded: number of corpora lutea, number of implantation sites, number of resorption sites (classified as early and late), number of live and dead fetuses, individual weights of live fetuses. Dead fetuses were defined as dead conceptuses showing distinct digits visible on fore and hind-paws. Intra-uterine resorption death was classified as early resorptions when macroscopic discrimination between fetal residues and placental material was not possible and late resorption when distinct macroscopic discrimination between fetal residues and placental material remains possible.

5. Fetal examination
All data were recorded without knowledge of treatment group. All live fetuses were killed by subcutaneous injection (0.1 mL/fetus) of Dolethal® and subjected to an external examination. After

internal examination of the neck, the head of fetuses from approximately half of each litter was immersed in Bouin's fluid and the internal structures examined after fixation. The bodies of all fetuses were dissected for soft tissue abnormalities and sexed. Fetuses were eviscerated, skinned, and fixed in absolute ethanol before staining. A modification of the Staples and Schnell staining technique was used and a subsequent skeletal examination was performed.

Structural deviations were classified as follows:
Malformations
A permanent structural change that is likely to adversely affect the survival or health.

Variations
A change that occurs within the normal population under investigation and is likely to adversely affect survival or health (this might include a delay in growth or morphogenesis that has otherwise followed a normal pattern of development).

6. Statistics
Means and standard deviations (STD) for all maternal, litter, and fetal parameters were calculated for each group. Statistical analyses were performed on the following variables using TERATEST Phase 1, Version 12 (maternal endpoints), TERATEST Phase 2, Version4 (litter based endpoints and fetal endpoints except fetal body weights analyzed using SAS programs (Version 9.2).

Maternal endpoints
Body weight change calculated according to time periods, calculated corrected body weight change.

Litter-based and fetal endpoints
Number of corpora lutea, number of implantation sites, number of resorptions (early and late), pre- and post-implantation loss percentages, fetal sex (described in terms of percent male fetuses), fetal death status (described in terms of number of dead fetuses and number of litters with dead fetuses), fetal body weight (combined sexes and per sex; mean fetal body weight per litter was the statistical unit).

For all parameters except fetal body weights, homogeneity of variances between control and treated groups was evaluated using the Bartlett test. If not significant, means were compared using the analysis of variance (ANOVA), which was followed by the Dunnett test (2-sided) if ANOVA indicated significance.

If the Bartlett test was significant (for body weight change, corrected body weight change, number of corpora lutea, number of implantation sites or number of resorptions parameters), group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance.

If the Bartlett test was significant, a log transformation (for food consumption, liver weight) or an arcsine root transformation (for pre- or post-implantation loss percentages) was performed. If the Bartlett test on transformed data was not significant, means were compared using the analysis of variance (ANOVA), which was followed by the Dunnett test (2-sided) if ANOVA indicated significance. If the Bartlett test on transformed data was significant, group means were compared using

the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance.

If the Bartlett test was significant for fetal sex (male *vs.* female fetuses) and fetal death status (live *vs.* dead fetuses) endpoints, control group and each exposed group were compared using the Chi-square test (for fetal sex parameter) or the Fisher Exact test (2-sided) (for fetal death status parameter). Death status was analyzed both using the fetus as the statistical unit and using the litter as the statistical unit.

For fetal body weight, homogeneity of variances between control and treated groups was evaluated using the Levene test. If not significant, means were compared using the analysis of variance (ANOVA), which was followed by the Dunnett test (2-sided) if ANOVA indicated significance. If the Levene test was significant a log transformation was performed. If the Levene test on transformed data was not significant, means were compared using the analysis of variance (ANOVA), which was followed by the Dunnett test (2-sided) if ANOVA indicated significance. If the Levene test on transformed data was significant, group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance.

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

The homogeneity of group variances, results of the ANOVA or the Kruskal-Wallis tests were evaluated at the 5% level of significance. Group means were compared at the 5% and 1% levels of significance.

7. Indices

Data from non-pregnant animals were not included in group mean calculations of any maternal parameters. Data from pregnant animals that died or were sacrificed were included in body weight and food consumption calculations up to the last day data was recorded.

The following indices (endpoints) were calculated for each dam:

- Maternal body weight (BW) changes for interval periods were calculated as follows:
  Body Weight Changes (GD 6 to 8) = BW on GD 8 - BW on GD 6
- Corrected body weight change (CBWC) was calculated as follows:
  CBWC = (BW on GD 29 - BW on GD 6) - (gravid uterine weight)
- Average food consumption (FC) was calculated during intervals in g/day, *e.g.*:
  Food Consumption (between GD 3 to 6) = (FC GD 4 + FC GD5 + FC GD6)/3
- Average liver weight (LW).

The following endpoints were calculated for each litter (dam):

- Pre-implantation loss was calculated per litter as a percentage according to the formula:
  [(Number of corpora lutea - Number of implantations)/Number of corpora lutea] x 100
- Post-implantation loss was calculated per litter as a percentage according to the formula:
  [(Number of implantations - Number of live fetuses)/Number of implantations] x 100
- Number of live fetuses was calculated as the sum of number of live fetuses per litter
- Number of dead fetuses was calculated as the sum of number of dead fetuses per litter
- Percentage of dead fetuses per litter was calculated according to the formula:

(Number of dead fetuses/Total number of fetuses) x 100

- Percentage of male fetuses per litter was calculated according to the formula:
  (Number of live male fetuses/Total number of live male and female fetuses) x 100
- Mean fetal body weight per litter was calculated according to the formula:
  Sum of individual weights of live fetuses/Number of weighed live fetuses
- Mean fetal body weight per litter and per sex was calculated according to the formula (example for male fetuses):
  Sum of individual weights of live male fetuses / Number of weighed live male fetuses

The following endpoints were calculated per group:

- Mean fetal body weight was calculated according to the formula:
  Sum of mean fetal body weight of live fetuses per litter / Number of litters with weighed live fetuses
- Mean fetal body weight per sex was calculated according to the formula (example for male fetuses):
  Sum of mean fetal body weight per litter of live male fetuses / Number of litters with weighed live male fetuses.

For external, visceral and skeletal fetal findings, the percentage of fetuses affected per group for a given parameter was calculated using the following formula:

- Percentage of fetuses affected per group:
  (Sum of live fetuses affected / Number of live fetuses examined) x 100
- The percentage of litters affected per group was calculated using the following formula:
  (Sum of litters with live fetuses affected / Number of litters with live fetuses examined) x 100.

II. Results and discussion

A. Maternal observations

1. Mortality

There were no treatment-related deaths at 40, 15 or 7.5 mg/kg/day.

2. Abortions

In the control group, one female aborted on GD 25. The macroscopic observation showed red foci on lungs and prominent lobulation of the liver.

3. Clinical signs:

No treatment-related clinical signs were observed at 40, 15 or 7.5 mg/kg/day; observed clinical signs were those commonly encountered in this strain of rabbit or were observed with no dose-relationship.

4. Body weight

At 40 mg/kg/day, there was a mean body weight loss of 0.01 g in all pregnant females between GD 6 and 8 compared to a mean body weight gain of 0.02 g in the controls; this effect was considered to be treatment-related, though it was not statistically significant and remained within the range of in-house Historical Control Data (HCD), since mean maternal body weight between GD 6 and 10 was lower than the controls (- 67%, $p \leq 0.05$). Thereafter, mean body weight gain was comparable to the controls

throughout all intervals. Mean maternal body weights were comparable to the controls while mean maternal corrected body weight change (maternal body weight change between GD 6 and 29 - gravid uterine weight) was 12% lower than the controls (not statistically significant) and slightly outside the range of in-house HCD.

At 15 and 7.5 mg/kg/day, there were no treatment-related effects on mean maternal body weights, body weight gains and maternal corrected body weight change. The few statistically significant increases in mean body weight gain noted at both dose levels were observed in isolation and/or with no dose-relationship and were thus considered to be incidental.

Body weight gain data are summarized below in the following table.

Table 5.6.11-02: Mean (± SD) maternal body weight gain (g)

Interval	Dose level of BYI 02960 (mg/kg/day)			
	0	7.5	15	40
Number of pregnant dams	23	22	23	22
Pretreatment, GD 3-6:	0.03 (± 0.076)	0.03 (± 0.073)	0.01 (± 0.075)	0.02 (± 0.070)
Treatment, GD 6-8:	0.02 (± 0.042)	0.02 (± 0.035)	0.00 (± 0.031)	- 0.01 (± 0.060)
Treatment, GD 8-10:	0.04 (± 0.046)	0.04 (± 0.033)	0.03 (± 0.025)	0.03 (± 0.039)
Treatment, GD 10-14:	0.03 (± 0.081)	0.09 (± 0.056)**	0.07 (± 0.049)*	0.06 (± 0.054)
Treatment, GD 14-18:	0.03 (± 0.055)	0.05 (± 0.054)	0.07 (± 0.045)	0.04 (± 0.049)
Treatment, GD 18-22:	0.04 (± 0.046)	0.05 (± 0.046)	0.03 (± 0.042)	0.06 (± 0.061)
Treatment, GD 22-26:	0.06 (± 0.080)	0.04 (± 0.067)	0.04 (± 0.050)	0.07 (± 0.058)
Treatment, GD 26-29:	0.06 (± 0.069)	0.04 (± 0.059)	0.03 (± 0.049)	0.04 (± 0.076)
Treatment, GD 6-29	0.28 (± 0.228)	0.31 (± 0.111)	0.28 (± 0.160)	0.28 (± 0.116)
Corrected BW change	- 0.25 (± 0.177)	- 0.24 (± 0.110)	- 0.25 (± 0.157)	- 0.28 (± 0.126)

* : Statistically different ($p \leq 0.05$) from the control
** : Statistically different ($p \leq 0.01$) from the control

5. Food consumption

At 40 mg/kg/day, mean maternal food consumption was reduced by 20% between GD 6 to 8 ($p \leq 0.01$) and by 11% between GD 8 to 10 (not statistically significant). Thereafter, mean maternal food consumption was similar to the controls.

At 15 and 7.5 mg/kg/day, mean maternal food consumption was similar to the controls. A statistically significant increase in mean maternal food consumption was noted at both dose levels between GD 14 to 18 but was observed in isolation and with no dose-relationship and thus considered to be incidental.

6. Maternal necropsy findings and liver weights

At 40, 15 and 7.5 mg/kg/day, mean liver weight was similar to the controls and there were no treatment-related macroscopic findings in dams at scheduled necropsy. The few macroscopic findings observed occurred in isolation and/or with no dose-relationship and were thus considered to be incidental.

B. Litter data

Pregnancy rate was unaffected by treatment. The pregnancy rate was 96% at 7.5 and 40 mg/kg/day and 100% in the control group and at 15 mg/kg/day.
No treatment-related changes were noted on litter parameters, including the number of live fetuses, the number of implant sites per dam, the percentages of pre- and post-implantation losses, the number of early and late resorptions, the fetal death status, the fetal body weight and the percentage of male fetuses.

The number of implant sites per dam at 40 mg/kg/day and the number of live fetuses at 40 and 7.5 mg/kg/day were slightly higher than in the control group. As these values were not statistically significantly different from the controls and were only marginally outside the range of in-house HCD, the changes were considered to be incidental.

Cesarean section data are summarized in the following table:

Table 5.6.11-03: Cesarean section observations

Observation	Dose Level of BYI 02960 (mg/kg/day)				Historical control range
	0	7.5	15	40	
Maternal data:					
No. Animals assigned	23	23	23	23	NA
No. Animals pregnant	23	22	23	22	NA
Pregnancy rate, %	100	96	100	96	NA
No. Animals non-pregnant	0	1	0	1	NA
Maternal wastage					
Total No. intercurrent death or sacrifice (pregnant & non pregnant)	0	0	0	0	NA
Total No. intercurrent death or sacrifice (pregnant)	0	0	0	0	NA
Total No. intercurrent death or sacrifice (non pregnant)	0	0	0	0	NA
No. premature delivery	0	0	0	0	NA
No. abortion	1	0	0	0	NA
Uterine data at scheduled sacrifice:					
Total No. corpora lutea[c]	253	280	265	283	NA
Corpora lutea / dam	11.5 ± 1.8	12.7 ± 1.9	11.5 ± 1.6	12.9 ± 2.6	10.79 - 13.21
Total No. implantations[c]	231	239	225	254	NA
Implantations / dam	10.5 ± 2.1	10.9 ± 2.3	10.2 ± 2.0	11.5 ± 2.9	9.10 - 10.95
Total No. litters[c]	22	22	23	22	NA
Total No. live fetuses[c]	213	223	216	228	NA
Live fetuses / dam[c]	9.7 ± 2.5	10.1 ± 2.1	9.4 ± 1.8	10.4 ± 2.4	8.23 - 9.90
Total No. dead fetuses	8	5	7	10	NA
Dead fetuses / dam[c], %	3.2 ± 6.8	1.0 ± 4.3	2.9 ± 5.4	3.8 ± 6.0	0.74 - 6.58
Total No. early resorptions[c]	9	9	9	13	NA
Total No. late resorptions[c]	3	1	3	3	NA
Early resorptions / dam	0.3 ± 0.8	0.4 ± 0.7	0.4 ± 0.5	0.6 ± 0.6	0.167 - 1.000
Late resorptions / dam	0.1 ± 0.5	0.1 ± 0.3	0.1 ± 0.5	0.1 ± 0.5	0.000 - 0.333
Litters with total resorptions[c, d]	0	0	0	0	NA
Pre-implantation loss per litter, %	9.2 ± 8.3	14.5 ± 13.6	11.0 ± 14.1	10.9 ± 11.0	9.08 - 26.73
Post-implantation loss per litter, %	8.7 ± 13.0	6.1 ± 9.2	7.4 ± 9.2	9.7 ± 9.5	4.07 - 19.25
Mean fetal weight, combined sexes, g	37.23 ± 6.20	38.07 ± 3.44	39.14 ± 4.26	37.89 ± 4.69	34.96 - 41.37
Mean fetal weight, males, g	37.31 ± 6.49	38.66 ± 4.05	39.48 ± 4.50	38.04 ± 5.77	34.92 - 42.33
Mean fetal weight, females, g	36.52 ± 6.86	37.60 ± 3.54	38.81 ± 4.41	37.75 ± 4.57	34.57 - 40.85
Sex ratio, % male	48.4 ± 18.6	52.4 ± 15.2	50.0 ± 16.4	47.4 ± 20.7	44.0 - 53.8

* : Statistically different ($p < 0.05$) from the control. ** Statistically different ($p < 0.01$) from the control

[c] : Statistical analysis was not conducted on this endpoint

[d] : Also includes litters with dead fetuses only or dead fetuses and resorptions

NA : Not applicable

C. Fetal necropsy findings

1. Fetal evaluation: external observations
There were no treatment-related malformations or variations noted at the fetal external examination.

Five fetuses in five different litters were observed with external malformations: three in the low dose group and two in the control group. As they were observed with no dose-relationship, these malformations were considered to have occurred spontaneously.

At 40 mg/kg/day, the incidence of the variation "eye bulge (uni/bi): protruding" was higher than in the control group and was outside the range of in-house historical control data (HCD) at the fetal level. However, as the observation was noted in only one litter and as the finding was not corroborated at the internal and skeletal observations, this increased incidence was considered to have occurred by chance.

The other variations were considered to be incidental, as they occurred in isolation or were observed with no dose-relationship and were well within the range of in-house HCD.

2. Fetal evaluation: visceral observations
There were no treatment-related malformations or variations noted at the fetal visceral examination.

The visceral malformations observed in a total of 18 fetuses were evenly distributed between the different dose groups including the control (6, 4, 4 and 4 malformed fetuses at 0, 7.5, 15 and 40 mg/kg/day, respectively) and did not have the same origin. They were thus considered to have occurred spontaneously.

The visceral variations observed occurred as isolated findings, were observed with no dose-relationship, and/or were within the range of in-house HCD. They were thus considered to be incidental.

3. Fetal evaluation: skeletal observations
There were no treatment-related malformations or variations noted at the fetal skeletal examination.
The skeletal malformations observed in a total of 7 fetuses were evenly distributed between the different dose groups including the control (2, 1, 2 and 2 malformed fetuses at 0, 7.5, 15 and 40 mg/kg/day, respectively). They were thus considered to have occurred spontaneously.

The incidence of the skeletal variations listed in the following table (Table 5.6.11-04) was higher than in the controls in at least the top dose group at the fetal and/or litter level. As the values were well within the in-house HCD and were not statistically significantly different from control, they were considered not to be treatment-related.

Table 5.6.11-04: Skeletal examinations

Dose level of BYI 02960 (mg/kg/day)	0	7.5	15	40	Historical Control Range	0	7.5	15	40	Historical Control Range
Observations #	Number of litters examined					Number of fetuses examined				
	22	22	23	22		213	223	216	228	
	Number of litters examined					Number of heads examined				
	22	22	23	22		112	117	115	119	
	Number of litters affected (% of litters affected)					Number of fetuses affected (% of fetuses affected)				
Variations Hyoid centrum: incomplete ossification or unossified.	9 (40.9)	10 (45.5)	11 (47.8)	12 (54.5)	(4.8 - 63.6)	20 (17.9)	13 (11.1)	16 (13.9)	23 (19.3)	(1.1 - 28.6)
5th and 6th sternebrae: incomplete ossification.	8 (36.4)	8 (36.4)	7 (30.4)	10 (45.5)	(14.3 - 45.8)	18 (8.5)	11 (4.9)	10 (4.6)	19 (8.3)	(2.1 - 12.6)
Pubis (uni/bi): incomplete ossification.	1 (4.5)	2 (9.1)	0 (0.0)	3 (13.6)	(0.0 - 27.3)	2 (0.9)	2 (0.9)	0 (0.0)	5 (2.2)	(0.0 - 4.3)
Insertion point (uni/bi) of pelvic girdle on 2nd sacral vertebra.	21 (95.5)	22 (100)	19 (82.6)	17 (77.3)	(66.7 - 95.8)	85 (39.9)	87 (39.0)	85 (39.4)	95 (41.7)	(28.2 - 56.7)

: Statistical analysis was conducted on the observations.
* : Statistically different ($p \leq 0.05$) from the control
** : Statistically different ($p \leq 0.01$) from the control

The other skeletal variations observed occurred as isolated findings, were observed with no dose-relationship, and/or were within the range of in-house HCD. They were thus considered to be incidental.

III. Conclusions

In conclusion, a dose level of 40 mg/kg/day BYI 02960 administered to the pregnant New Zealand White rabbit by oral gavage resulted in maternal toxicity as evidenced by body weight loss and significant reduced body weight gain and food consumption between GD 6 and 10 and lower mean maternal corrected body weight change in comparison to the controls. Fetal development was unaffected by treatment at any dose level tested. A dose level of 15 mg/kg/day was considered to be a NOAEL in terms of maternal toxicity, while a dose level of 40 mg/kg/day was considered to be a NOAEL in terms of developmental toxicity.

KIIA 5.7 - Neurotoxicity

All studies presented in this section were conducted between 2009 and 2011 and complied with the EU, OECD USEPA and Japanese MAFF testing guidelines and Good Laboratory Practice (GLP).

In an acute neurotoxicity study, technical grade BYI 02960 was administered by gavage in a single dose to non-fasted young adult Wistar rats at 0, 50, 200 and 800 mg/kg. Compound-related effects were observed at all dose concentrations in both sexes. Findings associated with treatment at the time-of-peak effect after dosing included piloerection, lower muscle tone, rapid respiration, low arousal, tremors, myoclonic jerks, chewing, repetitive licking of lips, gait incoordination, flattened or hunched posture, dilated pupils, impaired (uncoordinated or slow) righting reflex, impaired flexor and tail pinch responses and reduced rectal temperature. Automated measures of motor activity were also reduced in both sexes, compared to controls. The only treatment-related effects at 50 mg/kg were limited to higher incidences of piloerection in both sexes and dilated pupils in females only. A follow-up study was performed in order to establish a clear NOAEL for findings observed at all dose levels in the initial study. In this follow-up study, females only were used as they were equal or more sensitive than males at higher dose levels and were administered BYI 02960 at 20 or 35 mg/kg. No treatment-related effects were evident at either dose tested. The dose level of 35 mg/kg of BYI 02960 was considered to be the overall NOAEL for both sexes.

In a 90-day neurotoxicity study, through approximately 13 weeks of continuous dietary exposure to BYI 02960 at 100, 500 or 2500 ppm, there were no neurotoxic treatment-related findings apparent at any dietary level in either sex. Based on these findings, a NOAEL of 2500 ppm was established for the rat (equating to 143 and 173 mg BYI 02960/kg body wt/day for males and females, respectively).

In the developmental neurotoxicity study, BYI 02960 was administered via the diet from gestation Day (GD) 6 trhough lactation Day (LD) 21 to mated female Wistar rats at nominal concentrations of 0, 120, 500 or 1200 ppm with adjustment during lactation to maintain a more consistent dosage throughout the period of exposure. In maternal animals, body weight was statistically reduced at 1200 ppm on GD 20, LD 0 and LD 4, with non-statistical decreases that continued through termination on LD 21. In offspring at the same concentration, body weight was non-statistically reduced on PND 17 and 21 in both sexes. Body weight gain was statistically decreased in males and in both sexes combined for PND 4-17 and in males and females for PND 11-17. Startle amplitude was statistically increased in females on PND 60. Average session motor and locomotor activity was non-statistically increased in males on PND 13. There were no treatment-related effects in maternal animals or offspring at 500 and 120 ppm. Therefore, the NOAEL for both maternal animals and offspring is 500 ppm (42.4 mg/kg/day) in this study.

Table 5.7-01: Summary of neurotoxicity with BYI 02960

Type of study (Document N°) Doses	NO(A)EL (mg/kg/d)	LOAEL (mg/kg/d)	Adverse effects / target organs
Acute neurotoxicity in the rat M-415408-01-2 0, 20, 35, 50, 200 and 800 mg/kg bw	35 (M/F)	50 (M/F)	Piloerection and dilated pupils. At high dose levels: lower muscle tone, rapid respiration, gait incoordination, tremors, reduced motor activity, impaired righting reflex, impaired flexor and tail pinch responses
90-day neurotoxicity in the rat M-410022-01-2 0, 100, 500,2500 ppm	143/173 (M/F)	> 143/173 (M/F)	None
Developmental neurotoxicity in the rat M-434203-01-1 120, 500, 1200 ppm	42.4	102	↓ Body weight and body weight gain in dams ↓ Body weight and body weight gain in pups during lactation ↑ Startle amplitude (females only) on PND 60 ↑ Motor and locomotor activity on PND 13 (males only)

KIIA 5.7.1 - Acute neurotoxicity - rat

Report:	**KIIA 5.7.1/01, [redacted] J.-C.; 2011**
Title:	BYI 02960, An acute Neurotoxicity Study in the Rat by oral administration
Report No & Document No	SA 10096 M-415408-01-2
Guidelines:	OECD 424 (1997); EPA Health Effects Test Guideline (OPPTS 870.6200; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
GLP	Yes (certified laboratory)

Executive Summary

BYI 02960 (batch number 2009-000239: a beige powder, 96.2% w/w purity), an insecticide of the butenolide family, was administered once by oral gavage to separate groups of Wistar rats (12/sex/group) at dose levels of 0, 50, 200 and 800 mg/kg. Neurotoxicity assessment including a functional observational battery (FOB) and spontaneous motor activity was performed on 4 occasions (during pre-study phase, approximately 2 hours after dosing and then 7 and 14 days after dosing). All surviving animals were subjected to a complete necropsy. At least 6 animals/sex/group were subjected to neuropathological investigation with selected organs weighed and a range of organs fixed and examined microscopically.

A follow-up study was performed in order to establish a clear NOAEL for findings observed at all dose levels in the initial study. In this follow-up study, females only were used as they were equal or more sensitive than males at higher dose levels. Groups of 12 females were administered the test substance in similar conditions as in the initial study at dose levels of 0, 20 and 35 mg/kg. Neurotoxicity assessment

was limited to a partial FOB during pre-study and at the time-of-peak effect after dosing. All animals were sacrificed without necropsy examination.

Up to and including the highest dose tested of 800 mg/kg, there was no treatment-related effect in the terminal body weight and organ weights or at the macroscopic and microscopic examination.

At 800 mg/kg
One female died during neurobehavioral testing conducted at the time of peak effect after dosing. Before dying, this female had findings consistent with other females at this dose level as well as findings associated with moribundity. Another high-dose female was found dead on study day 5.
One week after dosing, mean absolute body weight gain was statistically significantly lower in both sexes (- 40% and - 46% in males and females, respectively, compared to controls). Body weight gain recovered after day 7 and overall was comparable to controls for the duration of the study.
Findings associated with treatment at the time-of-peak effect after dosing included piloerection, lower muscle tone, rapid respiration, low arousal, tremors, myoclonic jerks, chewing, repetitive licking of lips, gait incoordination, flattened or hunched posture, dilated pupils, impaired (uncoordinated or slow) righting reflex, impaired flexor and tail pinch responses and reduced rectal temperature. Automated measures of motor activity were also reduced in both sexes, compared to controls.
All effects were reversible, with none observed at later time points of the study.
There were no macroscopic or microscopic treatment-related observations in either sex.

At 200 mg/kg
At the time of peak effect after dosing, treatment-related observations at 200 mg/kg included piloerection, rapid respiration, gait incoordination and flattened body posture in both sexes, with a higher incidence of tremors in both sexes. In addition, automated measures of motor activity were reduced during the first 10-min interval of the session, while activity for the entire test session was comparable to controls.
All effects were reversible and were not observed at later time points of the study.

At 50 mg/kg
The only treatment-related effects at this dose were limited to higher incidences of piloerection (both sexes) and dilated pupils (females only) at the time of peak effect after dosing.

At 20 and 35 mg/kg
No treatment-related effects were evident at either dose tested in the follow-up study that was performed to establish a NOAEL for effects observed at 50 mg/kg.

As a conclusion and based on these results, the dose level of 35 mg/kg of BYI 02960 was considered to be the overall NOAEL for both sexes.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable in the vehicle at the room temperature for up to 28 days
2. Vehicle and /or positive control:	0.5% methylcellulose in water
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age:	7 to 8 weeks old
Weight at dosing:	
Initial study:	Mean group weight males between 298 g - 302 g; mean group weight females between 214 g - 216 g
Follow-up study:	Mean group weight females between 196 g - 197 g
Source:	[redacted] France
Acclimation period:	
Initial study:	At least 20 days
Follow-up study:	14 days
Diet:	A04C-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) *ad libitum* except at designated time periods
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum* except during neurobehavioral testing
Housing:	Animals were caged individually in suspended stainless steel wire-mesh cages
Environmental conditions:	Temperature: 22 ± 4 °C Humidity: 50 ± 20% Air changes: 10 to 15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates

Initial study: May 05 to June 11, 2010

Follow-up study: February 16 to March 03, 2011 at [redacted] France.

2. Animal assignment and treatment

There were 12 animals of each sex per dose group in the initial study. In this follow-up study, females (12 per dose group) only were used as they were equal or more sensitive than males at higher dose

levels. Animals were assigned using a randomization by weight. The test substance was administered by gavage as a single dose in 0.5% methylcellulose in deionized water, at a dosing volume of 10 mL/kg. The table below summarizes the study design.

Table 5.7.1-01: Study design and animal assignment

Initial study	Dose Group (mg/kg bw)			
Experimental Parameter	**Control**	**50**	**200**	**800**
Total number of animals/sex/group	12	12	12	12
Behavioral Testing (FOB, Motor Activity)[a]	12/sex	12/sex	12/sex	12/sex
Neuropathology [b]	6/sex	0/sex	0/sex	6/sex

[a] : FOB and motor activity were assessed prior to dosing and again during days 0, 7 and 14
[b] : Tissues from the mid- and low-dose groups were not examined, as no treatment-related neuropathology was
noted at the highest dose level

Follow up study	Dose Group (mg/kg bw)		
Experimental Parameter	**Control**	**20**	**35**
Total number of animals/group (Females only)	12	12	12
Behavioral Testing (FOB, Motor Activity)[a]	12/sex	12/sex	12/sex
Neuropathology [b]	6/sex	0/sex	6/sex

[a] : FOB and motor activity were assessed prior to dosing and again during days 0, 7 and 14
[b] : Tissues from the mid- and low-dose groups were not examined, as no treatment-related neuropathology was
noted at the highest dose level

The rationale for dose selection was based on the results of an acute oral toxicity study in young adult female Wistar rats. In that study, six fasted female Wistar rats were administered an acute oral (gavage) dose of 2000 mg/kg as an aqueous suspension in 2% cremophor EL in demineralized water, at a dosing volume of 10 mL/kg. Animals were observed for mortality and clinical signs for at least 14 days after treatment. The test substance produced four mortalities out of six animals and clinical signs were decreased motility, tremors, piloerection, labor breathing and clonical cramps. The animals administered at 300 mg/kg survived to treatment and the clinical signs were limited to loud breathing. These results supported the use of a limit dose (2000 mg/kg) in the neurotoxicity study but provided no information to establish the time of peak effect.
To establish the time of peak effect a group of five female Wistar rats was given orally a single dose of BYI 02960 at a dose level of 1000 mg/kg in suspension in aqueous solution of methylcellulose 400 at 0.5%. Clinical signs were observed within 30 minutes after dosing including among others rapid respiration, flattened body and gait abnormalities. However, clinical signs were more pronounced 1 and 2 hours after dosing and included in addition tremors, piloerection, noisy respiration and stagerring gait in almost all animals. Thereafter, some of these signs were still observed with a tendency to decrease in intensity.

Based on these data, the dose levels selected for the initial study were 0, 50, 200 and 800 mg/kg for both sexes. Based on findings at all doses of the initial study (*i.e.* piloerection and pupil dilatation), a follow-up study was conducted to establish a NOAEL. In the follow-up study, additional dose levels of

20 and 35 mg/kg were tested. Only females were tested since they were equal or more sensitive than males at higher dose levels.

3. Test substance preparation and analysis

The test substance was suspended in an aqueous suspension of methylcellulose 400 (0.5%) to provide the required concentration. There was one preparation for each concentration for the study. When not in use, the formulations were stored at approximately +5 °C (± 3 °C).

Homogeneity Analysis

The homogeneity of BYI 02960 in the formulation was verified on the preparation at the lowest and highest concentrations of the initial study to demonstrate adequate formulation procedures. The homogeneity of BYI 02960 in the formulation was verified in the 2 concentrations of the follow-up study. All results of homogeneity were within 95 - 103% of nominal concentration.

Stability Analysis

The stability of the test substance in 0.5% methylcellulose 400 suspension, has been demonstrated in a previous study (SA 07025) at concentrations of 1 and 250 g/L for up to 28 days under similar conditions of usage and storage.

Concentration Analysis

The concentration of the test substance in the dosing formulation was verified for each dose level. The mean values obtained from the homogeneity check were taken as measured concentration. All results of concentration were within 95 - 101% of nominal concentration.

4. Statistics

The following parameters were analyzed: body weight parameters, body weight change parameters calculated according to time intervals, terminal body weight, absolute and relative organ weights parameters, spontaneous motor activity, grip strength, landing foot splay and rectal temperature parameters.

Mean and standard deviation were calculated for each group. All statistical analyses were carried out separately for males and females. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance.

C. Methods

1. Mortality and clinical observations

Animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). All animals placed on study were observed for clinical signs at least once daily. The nature,

onset, severity, duration and recovery of clinical signs were recorded. Cages and cage trays were inspected daily for evidence of ill health such as blood or loose feces.

2. Neurobehavioral examinations

Initial study

During pre-study phase and on study day 1 (approximately 2 hours after dosing), day 7 and day 14, a neurobehavioral assessment (FOB and motor activity) was performed. All surviving animals were individually tested (the order of animal testing was randomly determined) by the same technicians who were blind with respect to the animal's group assignment.

Functional Observational Battery

Functional Observational Battery included:

- Home cage observation: while the animal was in its home cage, observations were collected regarding posture, piloerection, involuntary motor movements, gait abnormalities, vocalizations or any abnormal behavior
- Observation during handling, including ease to remove from cage, reaction to being handled, muscle tone, eyelid, lacrimation, salivation, nasal discharge, staining or any other signs such as alopecia, emaciation, temperature upon touching ("cold to touch")
- Open-field observation: each animal was individually observed in an open field for 2 minutes for piloerection, respiration, arousal, gait abnormalities, posture, involuntary motor movements, stereotypic movements, vocalizations and number of rearings, urine and feces spots
- Reflex and physiologic observations/measurements included:
  - Pupil size
  - Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary constriction by focusing a narrow beam of light in the eyes)
  - Surface righting reflex (by putting the animal on its back and evaluating its ability/rapidity to reassume a normal standing position)
  - Corneal reflex (by touching the medial canthus with a fine object and observing the quick and complete closure of the eyelids)
  - Flexor reflex (by pinching the toes and evaluating the presence/strength of the flexor response of each hindlimb)
  - Auditory startle response (by evaluating the animal response to an auditory stimulus)
  - Tail pinch response (by pinching the tail with a forceps and evaluating the animal reaction)
  - Grip strength: the fore- and hindlimb grip strength of animals was measured quantitatively using a grip strength apparatus equipped with one pull and one push strain gauge (Bioseb, Chaville, France)
  - Landing foot splay: the animal was dropped from approximately 30 cm above a padded surface and hindlimb foot splay was marked, measured and recorded
  - Body weight
  - Rectal temperature

Exploratory motor activity

Animals were tested individually using an automated photocell recording apparatus (Imetronic, Bordeaux, France) designed to measure quantitatively spontaneous exploratory activity in a novel

environment. Exploratory activity was recorded during the first 60 minutes, with data collected at 10-minute intervals throughout the session.

Follow-up study
During pre-study phase and approximately 2 hours after dosing (time-of-peak effect) a neurobehavioral assessment was conducted to establish a NOAEL for effects observed at higher dose levels. The evaluation included home-cage observations, observations during handling, and open field observations as previously described. Open field observations were not evaluated, since these tests were not affected at higher doses. Pupil size and pupil reflex were observed for all surviving animals.

3. Body weight
Initial study
Each animal was weighed on study Day 1, prior to dosing and then weekly during the study period as part of the FOB. Additionally, animals were weighed before scheduled necropsy (terminal body weight).

Follow-up study
Each animal was weighed on study Day 1, prior to dosing.

4. Food consumption
Food consumption was not measured in this study.

5. Sacrifice and pathology
Initial study
Necropsy procedure

- Animals used for neuropathology

On study Days 15, 16 or 17, the first six animals at each dose level were selected for perfusion and tissue collection, with replacement of two animals which were inadequately perfused or died during anesthesia.
These animals were deeply anesthetized by inhalation of Isoflurane and then euthanized by exsanguination during intravascular perfusion with a fixative solution. Prior to anesthesia, the animals were injected with an intraperitoneal dose of heparin sodium (60 mg/kg). The perfusion via the left ventricle consisted of a flush with phosphate buffer, followed by the fixative solution (a solution of 4% formaldehyde and 1% glutaraldehyde in phosphate buffer).
The necropsy was limited to the external and neuropathological examination. Significant macroscopic findings were recorded and only macroscopic lesions from neural tissues were collected.

- Remaining animals

The remaining non-perfused animals were deeply anesthetized by inhalation of Isoflurane, euthanized by exsanguination and necropsied. The necropsy included the macroscopic examination of the external surfaces, all orifices and all major body cavities. Significant macroscopic lesions were recorded but not sampled.

Tissue collection

- Animals used for neuropathology

The entire carcass of the animal was post-fixed after preparation (brain, cervical and lumbar spinal cord were exposed after perfusion fixation by removal of the bone). The brain of perfused animals was weighed after post-fixation. No other organ was weighed.

The following tissues, except the eyes and optic nerves, were dissected after post-fixation:
- Brain
- Dorsal root ganglia and their spinal nerve roots from the cervical and lumbar swellings (bilateral)
- Eyes (bilateral)
- Gasserian ganglia (bilateral)
- Gastrocnemius muscle (unilateral)
- Optic nerves (bilateral)
- Peripheral nerves (sciatic, tibial and sural nerves) (bilateral)
- Spinal cord (cervical, thoracic and lumbar)
- Macroscopic lesions in neural tissue or skeletal muscle.

Fixative:
The collected samples were post-fixed in the fixative solution used for the perfusion (a solution of 4% formaldehyde and 1% glutaraldehyde in phosphate buffer), except the eyes and optic nerves which were fixed in Davidson's fixative.

- Remaining animals

No organs were collected.

Histotechnology and histopathology
All organ samples from all animals in all groups were processed and embedded in paraffin wax or glycol methacrylate.
Histological slides were prepared for tissues from control and high-dose animals of both sexes.
The brain was trimmed in a standard manner using a metal rodent brain matrix. The resulting 8 coronal sections were representative of the following major brain regions: olfactory bulbs, cerebral cortex, caudate putamen, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, pons and medulla oblongata.
Brain, spinal cord (cervical, thoracic, lumbar), eyes, optic nerves and gastrocnemius muscle were embedded in paraffin wax, sectioned at approximately 4 µm and stained with hematoxylin and eosin (H&E).
Dorsal root ganglia (including dorsal and ventral root fibers) from the cervical and lumbar swellings, gasserian ganglia, and peripheral nerves (sciatic, tibial and sural) were embedded in glycol methacrylate, sectioned at approximately 2 to 3 µm and stained with Lee's stain. For the dorsal root ganglia and peripheral nerves, only the left side was evaluated.

All the organs mentioned above from all animals in control and high-dose groups were examined using light microscopy. Since no evidence of neuropathological alterations was found in the high dose groups, no further analysis was performed.

Following the initial histopathological examination, a review of representative slides was performed by a second pathologist, according to standard operating procedures.

Follow-up study
All animals were sacrificed without necropsy.

II. Results and discussion

A. Observations

1. Clinical signs
There were no treatment-related clinical signs observed at the daily observations throughout the study in any treated group compared to the controls. More detailed observations were performed as part of the FOB on Day 1 (time-of-peak effect), 7 and 14.

2. Mortality
There was no mortality in males at any dose throughout the study.
In females at 800 mg/kg, one female died during neurobehavioral testing conducted at the time of peak effect after dosing. Observations for this animal included piloerection, low muscle tone and arousal, rapid respiration, tremors, myoclonic jerks, convulsions, dilated pupils, absence of pupil and flexor reflexes and uncoordinated or slow surface righting reflex. Also at this dose level, another female was found dead on study day 5.
There was no mortality in females at lower dose levels.

B. Body weight
Mean body weights were not affected at any dose level in either sex, compared to controls. However, at 800 mg/kg, mean absolute body weight gain 7 days after dosing was statistically significantly lower in males and surviving females (- 40% and - 46% in males and females, respectively; $p \leq 0.01$ or 0.05), compared to controls. During the second week, mean body weight gain was slightly higher than control, such that mean body weight gain for the duration of the study was comparable to controls.
At 200 and 50 mg/kg, mean body weight parameters were not affected in either sex.

Table 5.7.1.02: Group mean body weight (BW) and cumulative body weight gain (BWG) (g)

BYI 02960 Dose level (mg/kg)	0	50	200	800
Male				
Initial BW Day 1 (%C)	**298**	**298 (100)**	**299 (100)**	**302 (101)**
BW Day 7 (%C)	333	334 (100)	330 (99)	322 (97)
Final BW Day 14 (%C)	**371**	**365 (98)**	**362 (98)**	**368 (99)**
BWG Days 1 to 7 (%C)	35	36 (103)	31 (89)	21** (60)
Overall BWG Days 1 to 14 (%C)	**72**	**67 (93)**	**63 (88)**	**66 (92)**
Female				
Initial BW Day 1 (%C)	**214**	**216 (101)**	**216 (101)**	**214 (100)**
BW Day 7 (%C)	227	231 (102)	228 (100)	222 (98)

Final BW Day 14 (%C)	**244**	**242 (99)**	**243 (100)**	**242 (99)**
BWG Days 1 to 7 (%C)	13	14 (108)	11 (85)	7* (54)
Overall BWG Days 1 to 14 (%C)	**30**	**25 (83)**	**26 (87)**	**26 (87)**

% C : % vs control
** : Statistically different ($p \leq 0.01$) from the control
* : Statistically different ($p \leq 0.05$) from the control

C. Neurobehavioral assessment

1. Home cage observations

At the home cage observation on day 1, the only treatment-related clinical sign was piloerection, which was observed at higher incidences in males at 200 and 800 mg/ kg (7/12 and 9/12, respectively), compared to 4/12 in controls. In females, although not dose-related, higher incidences of piloerection were noted at 50 and 800 mg/kg (6/12 and 8/12, respectively), compared to 2/12 in controls. This finding was confirmed at the open field observation.
This effect was reversible since it was not observed again at the following FOB sessions on day 7 and 14. No other treatment-related effect was observed at any dose level at any time point.

2. During handling observations

At 800 mg/kg on day 1, the main observation noted was a higher incidence of animals considered "cold to touch" in both sexes (10/12 males and 9/12 females), compared to 0/12 in controls. This correlates well with the lower rectal temperature recorded during this session. Lower muscle tone was also observed in both sexes (5/12 males and 8/12 females, compared to 1/12 and 0/12 in controls, respectively) and soiled fur was observed in 1/12 male and 2/12 females, compared to 0/12 in controls. At 200 mg/kg, soiled fur was observed in 1 male and lower muscle tone was observed in 3/12 males and 1/12 females. There was no treatment related observation made at the FOB sessions on day 7 and 14. No treatment-related effects were observed up to 50 mg/kg.

3. Open field observation

At 800 mg/kg on day 1 compared to controls, higher incidences were observed in both sexes in a few observations including piloerection, rapid respiration, low arousal, tremor, myoclonic jerks, chewing, repetitive licking of lips, gait incoordination, flattened or hunched posture, and a lower mean number of rearings.

A lower incidence of similar observations was evident at 200 mg/kg in both sexes, including piloerection, rapid respiration, gait incoordination and flattened body posture. In addition, compared to controls, higher incidence of involuntary motor movements (*i.e.* tremors) was observed in both sexes.

At 50 mg/kg, the only observation was a slightly higher incidence of animals with piloerection in both sexes, compared to controls. In the follow-up study, no treatment-related changes were observed up to 35 mg/kg compared to controls. In this follow-up study, observations like Straub tail (recorded when the tail is elevated at more than 45° angle from the surface) and repetitive licking of lips were recorded in all groups, including controls. Since these observations were not dose related and were not observed at higher dose levels, they are considered not to be treatment-related.

All findings on day 1 were reversible, with no treatment-related effects at the FOB sessions on day 7 and 14.

Table 5.7.1-03: Summary of significant changes in open field observations during the initial study at time peak-effect

BYI 02960 Dose levels (mg/kg)	0	50	200	800
Male				
Piloerection	4/12	8/12	12/12	12/12
Rapid respiration	1/12	0/12	9/12	12/12
Tremors	0/12	0/12	4/12	8/12
Myoclonic jerks	0/12	0/12	1/12	5/12
Chewing	0/12	0/12	0/12	5/12
Convulsions	0/12	0/12	0/12	0/12
Gait incoordination	0/12	0/12	3/12	7/12
Rearing (mean ± S.D.)	7.2 ± 2.4	7.8 ± 3.4	4.3 ± 3.6	2.7 ± 2.8
Low arousal	1/12	1/12	6/12	11/12
Repetitive licking of lips	1/12	0/12	0/12	2/12
Flattened or hunched posture	3/12	2/12	8/12	11/12
Female				
Piloerection	4/12	7/12	11/12	12/12
Rapid respiration	0/12	0/12	6/12	12/12
Tremors	0/12	0/12	4/12	10/12
Myoclonic jerks	0/12	0/12	0/12	7/12
Chewing	0/12	0/12	1/12	4/12
Convulsions	0/12	0/12	0/12	1/12
Gait incoordination	0/12	0/12	5/12	5/12
Rearing	12.3 ± 4.6	10.7 ± 3.3	9.9 ± 4.6	2.8 ± 2.8
Low arousal	0/12	0/12	1/12	8/12
Repetitive licking of lips	0/12	1/12	3/12	8/12
Flattened or hunched posture	0/12	0/12	3/12	11/12

4. Sensory reactivity

At 800 mg/kg on day 1, higher incidences in dilated pupils, uncoordinated or slow righting reflex, and abnormal flexor and tail pinch responses were observed in both sexes, when compared to controls.
At 200 mg/kg on day 1, higher incidences of dilated pupils were observed in both sexes, compared to controls. In addition in males, uncoordinated or slow righting reflex was observed in 4/12 animals, compared to 0/12 in controls.
At 50 mg/kg on day 1, the only treatment-related effect observed was a higher incidence of dilated pupil in females.

At 20 and 35 mg/kg on day 1, there were no effects on the pupil size.

All signs were reversible, with no treatment-related findings at the FOB sessions on day 7 and 14. At 800 mg/kg on Day 14, 5/12 males showed uncoordinated or slow surface righting reflex however since

there was no change in this parameter of day 7, this observation was considered not to be treatment-related.

Table 5.7.1-04: Summary of significant changes in sensory reactivity during the initial study at time peak-effect

BYI 02960 Dose levels (mg/kg)	0	50	200	800
Male				
Right pupil: dilated	0/12	1/12	4/12	6/12
Left pupil: dilated	0/12	1/12	4/12	6/12
Incoordianted or slow surface righting reflex	0/12	0/12	4/12	6/12
Abnormal tail pinch response	1/12	4/12	2/12	4/12
Female				
Right pupil: dilated	1/12	6/12	7/12	10/12
Left pupil: dilated	1/12	6/12	7/12	10/12
Incoordianted or slow surface righting reflex	0/12	1/12	2/12	5/12
Abnormal tail pinch response	3/12	2/12	4/12	8/11

5. Grip strength, landing foot splay, rectal temperature and body weights

During the session conducted at the time of peak effect after dosing, a lower rectal temperature was observed in both sexes, compared to controls (35.3 °C *vs.* 36.8 °C in males and 35.0 °C *vs.* 37.5 °C in females; p≤0.01). This effect was reversible, with no significant difference from control on day 7 and 14 after dosing.

All other parameters (fore- and hind-limb grip strength, landing foot splay and body weight) were not affected in males or females, at any dose or time point.

6. Exploratory locomotor activity

At 800 mg/kg, the spontaneous motor activity for the 60-min test session was lower in both sexes, compared to control (- 50% and - 61% in males and females, respectively; p ≤0.01) during the assessment conducted shortly after dosing. This effect was most pronounced during the first 10-min interval, when the activity is the highest in control animals before habituation ensues. At 200 mg/kg, a statistically significantly lower activity was recorded during the first interval, while activity for the overall test session was comparable to the controls. There were no differences from control at lower dose levels.

For sessions conducted 7 days and 14 days after dosing, spontaneous motor activity in males and females was comparable to controls at all dose levels.

Table 5.7.1-05: Significant changes in motor activity during the initial study at time peak-effect

BYI 02960 Dose levels (mg/kg)	0	50	200	800
Male				
First interval (10 minutes) (mean ± S.D.)	107 ± 37	120 ± 37	64 ± 32 *	31 ± 17 *
Total activity (60 minutes) (mean ± S.D.)	194 ± 47	254 ± 118	163 ± 92	97 ± 29 *
Female				
First interval (10 minutes) (mean ± S.D.)	136 ± 31	111 ± 28	72 ± 29 *	57 ± 23 *
Total activity (60 minutes) (mean ± S.D.)	293 ± 156	272 ± 154	199 ± 125	115 ± 72 *

* : Statistically different ($p \leq 0.05$) from the control

D. Sacrifice and pathology

1. Terminal body weight and organ weights

No relevant changes were noted in terminal body weight and organ weights of treated animals when compared to controls.

2. Gross pathology

- Animals used for neuropathology

No treatment-related macroscopic findings were observed.

The few macroscopic findings noted in the nervous system were not dose-related and not correlated with any microscopic findings. They were thus considered to be incidental in origin.

- Remaining animals

No treatment-related macroscopic findings were observed.

Two high-dose females died before the end of the study. Animal UT4F2486 died during neurobehavioral testing on study Day 1. Animal UT4F2483 was found dead on Study Day 5. No relevant macroscopic findings were noted at necropsy for either animal, which supports acute toxicity as the cause of death.

3. Microscopic pathology

No treatment-related microscopic findings were observed.

The few microscopic findings noted were considered to be incidental in origin.

III. Conclusions

As a conclusion and based on these results, the dose level of 35 mg/kg of BYI 02960 was considered to be the overall NOAEL for both sexes.

KIIA 5.7.2 - Delayed neurotoxicity following acute exposure

BYI 02960 is not an organophosporous insecticide; therefore this type of study is not triggered.

KIIA 5.7.3 - 28-day delayed neurotoxicity

BYI 02960 is not an organophosporous insecticide; therefore this type of study is not triggered.

KIIA 5.7.4 - Subchronic neurotoxicity - rat - 90-day

Report:	**KIIA 5.7.4/01, [redacted] J.-C.; 2011**
Title:	BYI 0296090-day Neurotoxicity Study in the Rat by dietary administration
Report No & Document No	SA 09283 M-410022-01-2
Guidelines:	OECD 424 (1997); EPA Health Effects Test Guideline (OPPTS 870.6200; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
GLP	Yes (certified laboratory)

Executive Summary

BYI 02960 (batch number 2009-000239: a beige powder, 96.2% w/w purity), an insecticide of the butenolide family, was administered continuously via dietary administration to separate groups of Wistar rats (12/sex/group) at constant concentrations of 100, 500 and 2500 ppm (equating approximately to 5.7, 29.4, 143 mg/kg body weight/day in males and 6.9, 34.8, 173 mg/kg body weight/day in females) for at least 90 days. A similarly constituted group of 12 males and 12 females received untreated diet and acted as a control. Animals were observed for clinical signs daily, with body weight and food consumption measured weekly. A detailed physical examination was performed once during the acclimatization phase and weekly throughout the study. Neurotoxicity assessment, consisting of a Functional Observational Battery (FOB) and automated measurement of motor activity, was performed on all animals on 5 occasions (pre-study, Study weeks 2, 4, 8 and 13 - 14). Ophthalmological examinations were performed on all animals during the acclimatization phase and on all animals of all dose groups during Week 13. All animals were subjected to a complete necropsy (macroscopic examinations). At least 6 animals from each group were perfused for neuropathological investigation. The brain was weighed and a range of tissues from the nervous system was collected, fixed and examined microscopically.

Up to and including the highest dose tested of 2500 ppm, there was no treatment-related mortality, clinical signs or ophthalmological changes during the study and no treatment-related effects were observed in any of the neurotoxicology endpoints, including neuropathological examinations, in either sex.

<u>At 2500 ppm</u>

Body weight parameters were clearly affected in both sexes, especially during the first week of treatment, when mean body weight remained static in females and the mean body weight gain was 50% lower in males as compared to controls. Overall at the end of the study, mean body weight gain at this dose level represented 85% and 79% of the control values in males and females, respectively. Consequently, mean body weight remained lower compared to controls throughout the study in both sexes (- 7% to - 10% in males and - 5% to - 9% in females).

Mean food consumption was significantly lower in both sexes during the first week of treatment (- 18% and - 29% in males and females, respectively) and remained slightly lower in both sexes throughout the study (up to - 14% and up to - 13% in males and females, respectively, the effect being statistically significant on several occasions).
At necropsy, mean terminal body weight was 7% lower in both sexes, compared to controls. In remaining animals not used for neuropathology, enlarged liver was observed in 4/6 females.

At 500 ppm and 100 ppm
There were no treatment-related effects in either sex.

As a conclusion, dietary exposure to BYI 02960 for a minimum 13 weeks produced no evidence of neurotoxicity, at dietary levels as high as 2500 ppm (equivalent to 143 and 173 mg/kg bw/day in males and females, respectively); therefore, this is considered to be a NOAEL for neurotoxicology endpoints.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable at 70 and 2500 ppm in the diet at the room temperature for up to 110 days
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Rat
Age:	7 to 8 weeks old
Weight at dosing:	Mean group weight males between 294 g - 297 g; mean group weight females between 205 g - 211 g
Source:	[redacted] France
Acclimation period:	19 days
Diet:	A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) *ad libitum* except at designated time periods
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum* except during neurobehavioral testing
Housing:	Animals were caged individually in suspended stainless steel wire-mesh cages

Environmental conditions:

Temperature:	22 ± 4 °C
Humidity:	50 ± 20%
Air changes:	10 to 15 changes per hour
Photoperiod:	Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates

November 04, 2009 to February 26, 2010 at [redacted], France

2. Animal assignment and treatment

There were 12 animals of each sex per dose group. Animals were assigned using a randomization by weight. Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 5.7.4-01: Study design and animal assignment

BYI 02960 in ppm	**0**	**100**	**500**	**2500**
Achieved dosages in mg/kg/day (M/F)	**0**	**5.7/6.9**	**29.4/34.8**	**143/173**
Total number of animals/sex/group	12	12	12	12
Behavioral Testing (FOB, Motor Activity) [a]	12/sex	12/sex	12/sex	12/sex
Neuropathology [b]	6/sex	0/sex	0/sex	6/sex

[a]: FOB and motor activity were assessed prior to dosing and again during days 0, 7 and 14
[b]: Tissues from the mid- and low-dose groups were not examined, as no treatment-related neuropathology was noted at the highest dose level.

The rationale for dose selection was based on the results of a previous subchronic 90-day toxicity study in the rat (M-329048-03-2; KIIA 5.3.2/01) in which dietary administration of up to 2500 ppm was tested and resulted in reduced body weight, body weight gain and food consumption. This dose level produced effects on hematological and clinical chemistry parameters (platelet count, bilirubin, glucose, cholesterol and triglycerides) and tissue pathology (liver and thyroid). The NOAEL in this study was 500 ppm, with a LOAEL of 2500 ppm.

3. Test substance preparation and analysis

The test substance in acetone solution was incorporated into the diet to provide the required dietary concentrations of 100, 500 and 2500 ppm. For control groups, a control formulation was prepared by adding an equivalent volume of acetone into the diet. There were two preparations for the study. The first preparation of test substance formulations was prepared to cover the dietary requirements over approximately an 8-week period and the second to cover the dietary needs until the end of the study. When not in use, the diet formulations were stored at room temperature.

Homogeneity Analysis

The homogeneity of BYI 02960 in diet was verified on the first preparation at the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean values obtained from the

homogeneity check were taken as the measured concentration. All results of homogeneity were within 95-104% of nominal concentration.

Stability Analysis
The stability of BYI 02960 was verified at 70 and 2500 ppm in the diet for up to at least 110 days, when kept at ambient temperature which covered the period of storage and usage on this study (results reported in the study SA 08337).

Concentration Analysis
The dietary levels of the test substance were verified for each concentration on each preparation. The mean values obtained from the homogeneity check were taken as measured concentration. All results of concentration were within 91 - 102% of nominal concentration.

4. Statistics
The following parameters were analyzed: body weight parameters, body weight change parameters calculated according to time intervals, terminal body weight, absolute and relative organ weights parameters, spontaneous motor activity, grip strength, landing foot splay and rectal temperature parameters.
Mean and standard deviation were calculated for each group. All statistical analyses were carried out separately for males and females. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance.

C. Methods

1. Mortality and clinical observations
Animals were checked for morbundity and mortality twice daily (once daily on weekends or public holidays). All animals were observed for clinical signs at least once daily during the study. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, duration and recovery of clinical signs were recorded. Cages and cage trays were inspected daily for evidence of ill health, such as blood or loose feces.

2. Neurobehavioral examinations
Before study initiation and during study Weeks 2, 4, 8 and 13 or 14, a neurobehavioral assessment (FOB and motor activity) was performed. All surviving animals were individually tested (the order of animal testing was randomly determined) by the same technicians who were blind with respect to the animal's group assignment.

Functional Observational Battery
Functional Observational Battery included:

- Home cage observation: while the animal was in its home cage, observations were collected regarding posture, piloerection, involuntary motor movements, gait abnormalities, vocalizations or any abnormal behavior
- Observation during handling, including ease to remove from cage, reaction to being handled, muscle tone, eyelid, lacrimation, salivation, nasal discharge, staining or any other signs such as alopecia, emaciation, temperature upon touching ("cold to touch")
- Open-field observation: each animal was individually observed in an open field for 2 minutes for piloerection, respiration, arousal, gait abnormalities, posture, involuntary motor movements, stereotypic movements, vocalizations and number of rearings, urine and feces spots
- Reflex and physiologic observations/measurements included:
  - Pupil size
  - Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary constriction by focusing a narrow beam of light in the eyes)
  - Surface righting reflex (by putting the animal on its back and evaluating its ability/rapidity to reassume a normal standing position)
  - Corneal reflex (by touching the medial canthus with a fine object and observing the quick and complete closure of the eyelids)
  - Flexor reflex (by pinching the toes and evaluating the presence/strength of the flexor response of each hindlimb)
  - Auditory startle response (by evaluating the animal response to an auditory stimulus)
  - Tail pinch response (by pinching the tail with a forceps and evaluating the animal reaction)
  - Grip strength: the fore- and hindlimb grip strength of animals was measured quantitatively using a grip strength apparatus equipped with one pull and one push strain gauge (Bioseb, Chaville, France)
  - Landing foot splay: the animal was dropped from approximately 30 cm above a padded surface and hindlimb foot splay was marked, measured and recorded
  - Body weight
  - Rectal temperature

Exploratory motor activity

Animals were tested individually using an automated photocell recording apparatus (Imetronic, Bordeaux, France) designed to measure quantitatively spontaneous exploratory activity in a novel environment. Exploratory activity was recorded during the first 60 minutes, with data collected at 10 - minute intervals throughout the session.

3. Body weight

Each animal was weighed 3 times during the acclimatization period, on the first day of test substance administration, then at least weekly throughout the treatment period. Additionally, animals were weighed before scheduled necropsy (terminal body weight).

4. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. From these records the mean daily consumption was calculated. Food spillage was also noted.

The weekly mean achieved dosage intake in mg/kg/day for each week and for Weeks 1 to 13 combined was calculated for each sex.

5. Ophthalmological examination

During the acclimatization period, all animals were subjected to an ophthalmological examination. After instillation of an atropinic agent (Mydriaticum, Merck Sharp and Dohme), each eye was examined by means of an indirect ophthalmoscope. During Week 13 of the treatment period, all surviving animals from all dose groups were re-examined using the same procedure.

6. Sacrifice and pathology

Necropsy procedure

- Animals used for neuropathology

On study Days 93- 96, the first six animals at each dose level were selected for perfusion and tissue collection, with replacement of two animals which were inadequately perfused.
These animals were deeply anesthetized by inhalation of Isoflurane and then euthanized by exsanguination during intravascular perfusion with a fixative solution. Prior to the anesthesia, the animals were injected with an intraperitoneal dose of heparin sodium (60 mg/kg). The perfusion via the left ventricle consisted of a flush with phosphate buffer, followed by the fixative solution (4% formaldehyde and 1% glutaraldehyde in phosphate buffer).
The necropsy was limited to an external and neuropathological examination. Significant macroscopic findings were recorded and only macroscopic lesions from neural tissues or skeletal muscle were collected, if present.

- Remaining animals

The remaining non-perfused animals were deeply anesthetized by inhalation of Isoflurane, euthanized by exsanguination and necropsied. The necropsy included the macroscopic examination of the external surfaces, all orifices and all major body cavities. Significant macroscopic lesions were recorded but not sampled.

Tissue collection

- Animals used for neuropathology

The entire carcass of the animal was post-fixed after preparation (brain, cervical and lumbar spinal cord were exposed after perfusion fixation by removal of the bone). The brain of perfused animals was weighed after post-fixation. No other organ was weighed.

The following tissues, except the eyes and optic nerves, were dissected after post-fixation:

- Brain
- Dorsal root ganglia and their spinal nerve roots from the cervical and lumbar swellings (bilateral)
- Eyes (bilateral)

- Gasserian ganglia (bilateral)
- Gastrocnemius muscle (unilateral)
- Optic nerves (bilateral)
- Peripheral nerves (sciatic, tibial and sural nerves) (bilateral)
- Spinal cord (cervical, thoracic and lumbar)
- Macroscopic lesions in neural tissue or skeletal muscle.

Fixative:
The collected samples were post-fixed in the fixative solution used for the perfusion (a solution of 4% formaldehyde and 1% glutaraldehyde in phosphate buffer), except the eyes and optic nerves which were fixed in Davidson's fixative.

- Remaining animals

No organs were collected.

Histotechnology and histopathology
All organ samples from all animals in all groups were processed and embedded in paraffin wax or glycol methacrylate.

Histological slides were prepared for tissues from control and high-dose animals of both sexes.
The brain was trimmed in a standard manner using a metal rodent brain matrix. The resulting 8 coronal sections were representative of the following major brain regions: olfactory bulbs, cerebral cortex, caudate putamen, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, pons and medulla oblongata.

Brain, spinal cord (cervical, thoracic, lumbar), eyes, optic nerves and gastrocnemius muscle were embedded in paraffin wax, sectioned at approximately 4 µm and stained with hematoxylin and eosin (H&E).

Dorsal root ganglia (including dorsal and ventral root fibers) from the cervical and lumbar swellings, gasserian ganglia, and peripheral nerves (sciatic, tibial and sural) were embedded in glycol methacrylate, sectioned at approximately 2 to 3 µm and stained with Lee's stain. For the dorsal root ganglia and peripheral nerves, only the left side was evaluated.

All the organs mentioned above from all animals in control and high-dose groups were examined using light microscopy. Since no evidence of neuropathological alterations was found in the high dose groups, no further analysis was performed.

Following the initial histopathological examination, a review of representative slides was performed by a second pathologist, according to standard operating procedures.

II. Results and discussion

A. Observations

1. Clinical signs

There were no treatment-related clinical signs in males or females at any dietary level.

2 .Mortality

There was no mortality throughout the study.

B. Body weight

At 2500 ppm, mean body weight remained static in females during the first week of treatment, compared to a mean weight gain of + 22 g in controls. In males at this dose level, mean absolute body weight gain was also markedly lower during the first week, compared to controls (- 50%, $p \leq 0.01$). Thereafter, mean absolute body weight gains remained significantly lower in both sexes, as compared to controls, throughout the study (statistically significant on all occasions but one in males), overall representing 85% and 79% of the control value in males and females, respectively.
As a consequence, mean body weight remained lower, compared to controls, throughout the study in both sexes (- 7% to - 10%, statistically significant on 3 occasions, $p \leq 0.05$ in males and - 5% to - 9%; statistically significant in most occasions, $p \leq 0.01$ or $p \leq 0.05$ in females).

At 500 ppm and 100 ppm, mean body weight parameters were not affected by the treatment.

Table 5.7.4-02: Group mean body weight (BW) and cumulative body weight gain (BWG) (g)

BYI 02960 Dose levels (ppm)	0	100	500	2500
Male				
Initial BW (Day 1) (%C)	**296**	**296 (100)**	**297 (100)**	**294 (99)**
BW Week 1 (Day 8) (%C)	342	343 (100)	341 (100)	317 (93)
BW Week 4 (Day 29) (%C)	429	432 (101)	433 (101)	392* (91)
BW Week 8 (Day 57) (%C)	505	500 (99)	512 (101)	460 (91)
Final BW Week 13 (Day 92) (%C)	**563**	**561 (100)**	**569 (101)**	**521 (93)**
BWG Weeks 1-4 (Days 1 to 29) (%C)	134	135 (101)	136 (101)	99** (74)
BWG Weeks 1-8 (Days 1 to 57) (%C)	209	204 (98)	214 (102)	166* (79)
Overall BWG (Days 1 to 92) (%C)	**268**	**265 (99)**	**272 (101)**	**227 (85)**
Female				
Initial BW (Day 1) (%C)	205	211 (103)	210 (102)	208 (101)
BW Week 1 (Day 8) (%C)	227	231 (102)	229 (101)	207* (91)
BW Week 4 (Day 29) (%C)	260	267 (103)	269 (103)	246 (95)
BW Week 8 (Day 57) (%C)	289	291 (101)	296 (102)	269* (93)
Final BW Week 13 (Day 92) (%C)	**309**	**313 (101)**	**317 (103)**	**290 (94)**
BWG Weeks 1-4 (Days 1 to 29) (%C)	55	56 (102)	60 (109)	39** (71)
BWG Weeks 1-8 (Days 1 to 57) (%C)	83	80 (96)	86 (104)	61** (73)
Overall BWG (Days 1 to 92) (%C)	**104**	**103 (99)**	**107 (103)**	**82** (79)**

% C : % *vs.* control
** : Statistically different ($p \leq 0.01$) from the control
* : Statistically different ($p \leq 0.05$) from the control

C. Food consumption and achieved dosages

At 2500 ppm in both sexes, mean food consumption was significantly lower during the first week of treatment, compared to control (- 18% and - 29% in males and females, respectively; $p \leq 0.01$). Afterwards, mean food consumption remained slightly lower in both sexes, compared to controls, on most occasions (- 3% to - 14% and - 4% to - 13% in males and females, respectively); this difference being statistically significant on several occasions throughout the study ($p \leq 0.01$ or $p \leq 0.05$).

At 500 ppm and 100 ppm, mean food consumption was not affected by the treatment in either sex.

Table 5.7.4-03: Mean food consumption/day (FC) (g)

BYI 02960 Dose levels (ppm)	0	100	500	2500
Male				
Initial FC Week 1 (Day 8) (%C)	**26.8**	**26.9 (100)**	**26.9 (100)**	**21.9** (82)**
FC Week 4 (Day 29) (%C)	**25.9**	**25.9 (100)**	**27.9 (108)**	**25.0 (97)**
FC Week 8 (Day 57) (%C)	**26.1**	**24.3 (93)**	**26.7 (102)**	**24.9 (95)**
Final FC Week 13 (Day 92) (%C)	26.8	26.6 (99)	27.6 (103)	24.5 (91)
Female				
Initial FC Week 1 (Day 8) (%C)	**20.8**	**20.0 (96)**	**19.7 (95)**	**14.7 ** (71)**
FC Week 4 (Day 29) (%C)	**19.3**	**19.1 (99)**	**20.2 (105)**	**18.5 (96)**
FC Week 8 (Day 57) (%C)	**18.8**	**18.3 (97)**	**18.7 (99)**	**17.3 (92)**
Final FC Week 13 (Day 92) (%C)	18.8	19.3 (103)	19.0 (101)	18.1 (96)

% C : % *vs.* control
** : Statistically different ($p \leq 0.01$) from the control
* : Statistically different ($p \leq 0.05$) from the control

The mean achieved dietary intake of BYI 02960, expressed in mg/kg/day, was as follows:

Table 5.7.4-04: Mean achieved dietary intake of BYI 02960 (mg/kg/day)

Sex	Male				Female			
Nominal level (ppm)	**0**	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
Week period 1 to 13	-	5.7	29.4	142	-	6.9	34.8	173

D. Ophthalmological examination

There were no treatment-related abnormalities at any dose level in either sex.

E. Neurobehavioral assessment

1. Home cage observations

No relevant changes were recorded in either sex in any test group, as compared to the control group, at any time point.

2. During handling observations

No relevant differences were recorded in either sex between the test groups and the control group at any time point.

3. Open field observation

No relevant differences were recorded for any of the parameters in either sex between the test groups and the control group at any time point.

4. Sensory reactivity

No relevant changes were recorded in any of the reflexes and responses evaluated in either sex between any of the test groups and the control group at any time point. The few findings observed were considered to reflect normal inter-individual variation.

5. Grip strength, landing foot splay, rectal temperature and body weights

No treatment-related changes were observed in grip strength, landing foot splay, rectal temperature or body weight parameters in either sex in any of the test groups.

Compared to controls, the very few statistical differences observed (a slightly higher mean forelimb grip strength in males at 100 ppm and 500 ppm on Week 4 and a lower mean rectal temperature in males at 100 ppm and 2500 ppm on Week 8) were considered to be incidental and not related to treatment, since they were observed without dose-effect relationship and were not observed at any other time point.

6. Exploratory locomotor activity

No relevant changes were recorded in overall (*i.e.*, total session) motor activity in any test group, as compared to the control group, at any time point. In addition, the general pattern of motor activity within the test session (*e.g.*, habituation) in test groups was similar to the control group, with no evidence of a treatment-related effect at any dose level.

D. Sacrifice and pathology

1. Terminal body weight and organ weights

At 2500 ppm, a lower mean terminal body weight was observed in both sexes (-7%, not statistically significant), when compared to controls, and was considered to be treatment-related.

At 2500 ppm in females, statistically-significantly higher mean brain-to-terminal body weight ratio was observed (+ 13%, $p \leq 0.01$). This finding was considered to be related to the lower mean terminal weight and not directly treatment-related.

2. Gross pathology

- Animals used for neuropathology

No treatment-related macroscopic findings were observed.

The few macroscopic findings noted in the nervous system were not dose-related and not correlated with any microscopic findings. They were thus considered to be incidental in origin.

- Remaining animals

At 2500 ppm, enlarged liver was observed in 4/6 females. This finding was consistent with the results from a previous 90-day study (M-329048-03-2, KIIA5.3.2./01) and is considered to be treatment-related.

3. Microscopic pathology

No treatment-related microscopic findings were observed. The few microscopic findings noted were considered to be incidental in origin.

III. Conclusions

As a conclusion, dietary exposure to BYI 02960 for a minimum 13 weeks produced no evidence of neurotoxicity, at dietary levels as high as 2500 ppm (equivalent to 143 and 173 mg/kg bw/day in males and females, respectively); therefore, this is considered to be a NOAEL for neurotoxicology endpoints.

KIIA 5.7.5 - Postnatal developmental neurotoxicity

Report:	**KIIA 5.7.5/01, [redacted]R.; 2012**
Title:	A Developmental Neurotoxicity Study with Technical Grade BYI 02960 in Wistar Rats
Study No & Document No	11-D72-UW M-434203-01-1
Guidelines:	OECD 426 (2007); EPA Health Effects Test Guideline (OPPTS 870.6300; 1998)
GLP	Yes (certified laboratory)

Executive Summary

BYI 02960 (batch number 2009-000239: a beige powder, 96.2% w/w purity), an insecticide of the butenolide family, was administered via the diet from gestation Day (GD) 6 through lactation Day (LD) 21 to mated female Wistar rats at nominal concentrations of 0, 120, 500, or 1200 ppm with adjustment during lactation to maintain a more consistent dosage throughout the period of exposure. The average mean daily intake of the test substance (mg BYI 02960/kg body wt/day) based on the average dietary consumption for the last two weeks of gestation and three weeks of lactation at 120, 500, or 1200 ppm, respectively, was 0, 10.3, 42.4, and 102 mg/kg/day. All test diets (including control) were provided for ad libitum consumption throughout the study, except during neurobehavioral testing. The Parental (P)-generation females were evaluated by cage-side and detailed clinical observations, body weight, food consumption, and reproductive endpoints. On postnatal Day (PND) 4, litters with a minimum of seven pups, including at least three per sex, were culled to yield, as closely as possible, four males and four females. Subsets of surviving offspring, representing 19–20 litters per dietary level, were subjected to evaluation using the following observations and measurements: detailed clinical observations and a detailed observational battery, pupil response, surface righting, balanopreputial separation or vaginal patency, body weight, food consumption, automated measures of activity (figure-eight maze), auditory startle habituation, learning and memory (passive avoidance after weaning and a water maze task beginning on PND 60 ± 2 days), and an ophthalmic examination. Neural tissues were collected from 10 rats/sex/dietary level (representing 20 litters) on PND 21 (brain only) and at study termination (approximately 75 days of age) for microscopic examination and morphometry.

Up to and including the highest dose tested of 1200 ppm, there was no treatment-related mortality, clinical signs or ophthalmological changes during the study and no treatment-related effects were observed in any of the reproductive parameters or neuropathological endpoints in either sex.

At 1200 ppm

In maternal animals, body weight was statistically reduced (-4% to -7%) on GD 20, LD 0, and LD 4, with non-statistical decreases (-4% to -6%) that continued through termination on LD 21. Body weight gain was non-statistically reduced (-21%) from GD 0–20.

In offspring, body weight was non-statistically reduced (-4% to -5%) on PND 17 and 21 in both sexes. Body weight gain for PND 4–17 was statistically decreased in males (-7%) and in both sexes combined (-6%). Body weight gain on PND 11–17 was statistically decreased in males (-10%), females (-7%), and in both sexes combined (-8%). There were similar non-statistical trends in body weight gain for PND 4–17 in high-dose females (-6%), for PND 4–21 and PND 11–21 in both sexes and in both sexes combined (-5% to -6%) as well. Startle amplitude for all 50 trials, as well as for the last 4 blocks of

trials, was statistically increased in females on PND 60. Average session motor and locomotor activity was non-statistically increased in males on PND 13.

At 500 ppm and 120 ppm
There were no treatment-related effects in maternal animals or offspring.

Dietary exposure to BYI 02960 from GD 6 through LD 21 to mated female Wistar rats resulted in treatment-related effects evident only at the HDT of 1200 ppm (102 mg/kg bw/day) in maternal animals and offspring. Therefore, the NOAEL for both maternal animals and offspring is 500 ppm (42.4 mg/kg bw/day) in this developmental neurotoxicity study.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable at 70 and 2500 ppm in the diet at the room temperature for up to 110 days
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Rat
Age:	12 (females) or 15 (males) weeks of age at co-housing
Weight (at ± 20% weight determination):	176.2–225.6 g range for females placed on study; Males had no specified weight requirements.
Source:	[redacted] NC
Acclimation period:	July 5, 2011 (receipt)–July 11, 2011 (released for study)
Diet:	Purina Mills Certified Rodent Diet Meal 5002 provided for *ad libitum* consumption during the acclimation period and throughout the study, except during neurobehavioral testing.
Water:	Tap water (Kansas City Missouri Municipal Water), *ad libitum* except during neurobehavioral testing
Housing:	Suspended stainless steel cages; individually, except during co-habitation (one male with one female) with deotized cage board in the bedding tray; individually (or with litters) in plastic cages with corn cob bedding during gestation and lactation. Each cage contained a feeder and source of water (pressure-activated water lixits and/or water bottles).

Environmental conditions:	Temperature:	22 ± 4 °C
	Humidity:	50 ± 20% (relative)
	Air changes:	Minimum daily average of 14.23 (animal Room 301), 12.85 (testing Room 302) and 13.96 (testing Room 304) air changes per hour
	Photoperiod:	Alternating 12-hour light and dark cycles (6 am - 6 pm); lights toggled off during ophthalmic examinations

B. Study Design

1. In life dates – July 5, 2011 to October 21, 2011

2. Animal assignment and treatment

Upon receipt, P-generation females were examined and those considered acceptable were placed into individual cages and acclimated to their ambient laboratory conditions for at least six days prior to being placed on study. Male animals were randomly assigned an identification number. For the holding period, animal care personnel observed the animals at least once daily for moribundity and mortality. All planned or unplanned activities associated with either the animals or their rooms, as well as changes in the status of either the animals or their room, were documented. With completion of the acclimation period, a veterinarian reviewed the status of the animals prior to their release for study. Dams were assigned to detailed observational testing as shown in the Study Design Table.

P-generation females were weighed assigned to the control or an exposure group in sequence, as they were determined to be inseminated. This approach was used to ensure unbiased assignment of the animals to dose groups and an approximately equal number of litters from each dose group available for testing on a given day. Females not placed on study were sacrificed without necropsy. Animals were assigned an identification number that specifies the sex, dietary level, cage number, and identifies it with the study. P-generation males served only as breeders. As such, they had no specific weight requirements and were arbitrarily selected for co-housing with females.

P-generation males and females were identified by cage card and tail mark (males) or tail tattoo (females). F_1-generation animals that were born alive were identified by tattoo; pups found dead were identified with a marking pen.

Offspring were assigned to testing subgroups at the time of litter standardization on PND 4. An animal allocation program written in SAS was used to assign offspring to one of the following four sets (designated A–D) for assessment at each age. One male and/or female per litter [approximately 16–20 (minimum 10)/sex/dietary level, representing at least 20 litters per level]: motor activity (Set A), auditory startle (Set B), passive avoidance, water maze, and detailed observational battery (Set C)[1]. On PND 21, the whole brain was collected from a separate group of randomly selected offspring (Set D;

[1] Only 19 litters were represented at each dietary level for passive avoidance and water maze. The number of litters represented for each test device is sufficient for establishing any test substance-related effects.

10/sex/dietary level; representing 20 litters per level) for micropathologic examination and morphometric analysis[2]. The remaining pups assigned to Set D (~10/sex/dietary level) were reserved for use as replacement animals or otherwise sacrificed on PND 21 without necropsy examination.

At approximately 50–60 days of age, randomly selected animals (a minimum of 10/sex/dietary level, representing at least 20 litters per level) from Sets A, B, and C were subjected to an ophthalmologic examination. At termination on PND 75 (±5 days), these animals were anesthetized and sacrificed by perfusion, with neural and muscle tissues collected for micropathologic examination and morphometric analysis. At termination on PND 75 (±5 days), brains were collected from additional randomly selected animals (10/sex/dietary group; representing 20 litters per level)[3]. These brains were weighed (fresh tissue weight) and then discarded.

The remaining animals assigned to sets A–C were sacrificed without routine gross necropsy examination or collection of tissues.

Table 5.7.5-01: Study design and animal assignment

Experimental Parameter	Dietary Level			
	Control	120 ppm	500 ppm	1200 ppm
Maternal Animals				
No. of Maternal Animals Assigned	**30**	**30**	**30**	**30**
Detailed Observational Battery (GD 13, 20/LD 11, 21)	**30/10**	**30/10**	**30/10**	**30/10**
Offspring				
Set C; Detailed Observational Battery [PND 4, 11, 21, 35(±1), 45(±1), 60(±2)]	**20 (min. 10)/sex**	**20 (min. 10)/sex**	**20 (min. 10)/sex**	**20 (min. 10)/sex**
Set A; Motor Activity [PND 13, 17, 21, 60(±2)]	**20 (min. 10)/sex**	**20 (min. 10)/sex**	**20 (min. 10)/sex**	**20 (min. 10)/sex**
Set B; Auditory Startle Habituation [PND 23, 60(±2)]	**20 (min. 10)/sex**	**20 (min. 10)/sex**	**20 (min. 10)/sex**	**20 (min. 10)/sex**
Set C; Learning and Memory [PND 23/30, 60 (±2)/67(±2)]	**16 (min. 10)/sex**	**16 (min. 10)/sex**	**16 (min. 10)/sex**	**16 (min. 10)/sex**
Set D; Brain Weight				
PND 21	**10/sex**	**10/sex**	**10/sex**	**10/sex**
PND 75(±5)	**10/sex**	**10/sex**	**10/sex**	**10/sex**
Set D; Neuropathology[a]				

[2] Only 19 litters were represented at the 1200 ppm dietary level. Two brains were inadvertently collected from littermates in the litter from dam UW3123.

[3] Only 9 females and 19 litters were represented at the 1200 ppm dietary level. The brain for one female was inadvertently not weighed at termination.

Experimental Parameter	Dietary Level			
	Control	120 ppm	500 ppm	1200 ppm
PND 21	**10/sex**	**10/sex**	**10/sex**	**10/sex**
PND 75(±5)	**10/sex**	**10/sex**	**10/sex**	**10/sex**

[a]: Tissues from the mid- and low-dose groups were not examined, as no treatment-related neuropathology was noted at the highest dose level

Doses were selected based on the results obtained from a two-generation reproduction study conducted with this test substance (M-417665-01-2; KIIA5.6.1/02). In that study, significant body weight and body weight gain decreases were observed throughout the study in females, as well as significant birth and body weight decreases in F_1 pups and adults at the top dose of 1800 ppm. A decrease in body weight gain (-20.5%) in P-generation females at the mid-dose of 500 ppm was the only relevant significant finding at this dose level.

3. Test substance preparation and analysis

The test substance in acetone solution was incorporated into the diet to provide the required dietary concentrations of 120, 500 and 1200 ppm. Concentrations of the test substance in the diet were measured by HPLC-UV, using five batches of feed that were used in this study. Formulations were prepared weekly by mixing appropriate amounts of the test substance in the diet (Purina Mills Certified Rodent Diet Meal 5002) and stored at freezer (average temperature -22 ± 4 °C) conditions. Dietary concentrations were adjusted (reduced) during lactation, relative to gestation, to maintain a more constant dosage (mg/kg/day) throughout the period of exposure and to compensate for the increase in food consumption by dams during lactation. Dietary levels during gestation were 0, 120, 500, or1200 ppm and were then reduced during Weeks 1–3 of lactation by 50% to offset the substantial increase in food consumption that is normally observed in dams during lactation, as noted in table 5.7.5-02. For control groups, a control formulation was prepared by adding an equivalent volume of acetone into the diet.

Table 5.7.5-02: Adjustment of Dietary Concentration of BYI 02960 During Lactation

Feeding Period	Dietary Concentrations (ppm)			
Gestation	**0**	**120**	**500**	**1200**
Lactation	**0**	**60**	**250**	**600**

Homogeneity and Stability Analysis

The homogeneity (95 – 98% of nominal concentrations) and stability of the test substance in rodent feed was verified at dietary concentrations of 20 and 2500 ppm and were determined to be homogeneous and stable for seven days at room temperature (average temperature 22 ± 4 °C) and 28 days at freezer conditions (average temperature -22 ± 4 °C).

Concentration Analysis

During gestation, the nominal 120, 500, and 1200 ppm dietary levels averaged 98–102% of the nominal concentrations. Based on these results, the average dietary levels during gestation were 0, 119, 508, and 1178 ppm, respectively. For lactation, dietary levels were reduced 50% to achieve a more consistent dosage (mg/kg bw/day) throughout the period of exposure, since food consumption increases during this time period. During lactation, the nominal 60, 250, and 600 ppm dietary levels averaged 98% of

the nominal concentrations. Based on these results, the average dietary levels during lactation were 0, 58.6, 244, and 588 ppm, respectively.

C. Methods

1. In-life observations

a. Maternal animals:

1) Clinical Observations

Following acclimation and continuing until animals were removed from the study (females only) or completion of co-housing (males only), P-generation males and females were observed (cage-side) for clinical signs at least once daily[4]. These observations were sufficient to characterize mortality, moribundity, behavioral changes, and overt toxicity by viewing the animal in its cage. At the discretion of the observer, animals were removed from the cage for a more detailed examination.

2) Detailed Observations

A detailed evaluation of the dams for clinical signs with a physical examination was conducted once daily from the initiation of exposure (GD 6) through LD 21. These observations were performed by an individual who was aware of the animal's dosage group assignment.

3) Detailed Observational Battery

Animals that were presumed to be pregnant (approximately 30 per dietary level) were observed on GD 13 and GD 20 and a minimum of 10 dams/dietary level that were maintained on study with suitable litters were also observed on LD 11 and LD 21. All observations were performed by individuals who were unaware of each animal's dose group assignment. This evaluation was performed under standard animal room conditions (temperature, relative humidity, etc.) and included observations in the home cage, during handling, and outside the home cage in an open field, using standardized procedures. This observational battery included, but was not limited to, assessments (with severity scoring) of lacrimation, salivation, piloerection, exophthalmia, urination, defecation, pupillary function, palpebral closure, convulsions, tremor, abnormal movements, unusual behaviors, and posture and gait abnormalities.

The checked (X) observations were evaluated:

[4] Mortality checks were not documented for 13 August 2011.

DETAILED OBSERVATIONS	
X	Signs of autonomic function, including: 1) Ranking of degree of lacrimation and salivation, with range of severity scores from none to severe 2) Presence of absence of piloerection and exophthalmos 3) Ranking or count of urination and defecation, including polyuria and diarrhea 4) Pupillary function such as constriction of the pupil in response to light, or a measure of pupil size 5) Degree of palpebral closure, e.g., ptosis
X	Description, incidence, and severity of any convulsions, tremors, or abnormal movements
X	Description and incidence of posture and gait abnormalities
X	Description and incidence of any unusual or abnormal behaviors, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may have facilitated interpretation of the data

4) Body Weight and Food Consumption

Body weight and food consumption were measured once weekly during gestation and lactation, as follows: GD 6–13, GD 13–20, LD 0–7, LD 7-14, and LD 14–21. In addition, dams were weighed on GD 0 and LD 4. Measures of food consumption may have included consumption by the pups, especially during the third week of lactation. Fresh feed and clean feeders were provided weekly.

5) Delivery and Culling

Each dam was evaluated daily for evidence of delivery from GD 20 to the completion of delivery, which was designated LD 0 for the dam and PND 0 for the pups. Litter size (the number of pups delivered) and pup "status" at birth were recorded for each litter. Dams that delivered fewer than three pups per sex or litter size of fewer than seven pups were sacrificed (along with their litter) without necropsy examination. For litters that met the minimum size requirements, the size of each litter was adjusted on PND 4 to yield, as closely as possible, four males and four females. When the number of male or female pups was less than four, a partial adjustment was made (e.g., three females and five males). When there were more than 23 acceptable litters for any dietary level, the surplus litters were sacrificed on PND 4 after weighing without routine necropsy, with preference given to retaining litters with a full complement of four males and four females. Culled dams and pups were sacrificed by carbon dioxide (CO_2) asphyxiation and decapitation, respectively. Dams with insufficient litters were also sacrificed by CO_2 asphyxiation.

6) Moribund Animals and Animals Found Dead

There were no P generation females found moribund or dead while on study.

7) Termination

Males: Following co-habitation, males were sacrificed by CO_2 asphyxiation and discarded (with the exception of selected animals that were used for training or other studies).

Females: Dams were sacrificed on LD 21, by CO_2 asphyxiation, following the weaning of their respective litters. Females that were sperm positive and/or had an internal vaginal plug, but did not deliver, were sacrificed on GD 24 without necropsy examination.

b. Offspring

1) Litter Observations

The day of completion of parturition was designated as LD (PND) 0. As soon as possible following parturition, pups were examined for ano-genital distance to establish their gender, and then tattooed and weighed. Live pups were counted, sexed, and weighed individually for each litter on PND 0, 4, 11, 17, and 21. Daily throughout lactation, offspring were examined cage-side for gross signs of mortality or moribundity. Any gross signs of toxicity in the offspring were recorded as they were observed, including the time of onset, degree, and duration. More detailed observations for clinical signs were made once daily (a.m.) before weaning and once weekly thereafter. These observations were performed by an individual who was aware of assignments to dose level.

2) Developmental Landmarks

Beginning on PND 4, selected males and females were examined daily for surface righting, with the age of onset recorded. On PND 21, all pups were tested for the presence of pupil constriction.[5] Beginning on PND 29, female offspring were examined daily for vaginal patency and beginning on PND 38, male offspring were examined daily for balanopreputial separation.

3) Postweaning Observations

After weaning on PND 21, offspring were examined twice daily for mortality (except on weekends and holidays when only one check was performed), with detailed observations for clinical signs performed once weekly.

4) Body Weight and Food Consumption

Surviving pups were weighed on PND 0, 4, 11, 17, and 21, and once weekly thereafter. The individual pups were also weighed when vaginal patency or balanopreputial separation was first evident. Food consumption was not measured after weaning on PND 21, when all animals received untreated diet.

5) Neurobehavioral Evaluations

Observations and the schedule for those observations are summarized as follows. The test room used for motor activity, auditory startle habituation, and passive avoidance conditioning was a standard animal room set to be maintained on the same light/dark cycle as the room in which animals were housed, with tests conducted during the light phase. The water maze testing was performed in the room where animals were housed. The order of testing and assignment of animals to specific test devices was semi-random, such that groups were balanced across test times and devices and no animal was tested more than once in the same device[6]. One exception was that animals were purposely tested in the same water maze on both occasions, as per standard procedure. Males and females were tested on the same days at the appropriate days of age. After sexual maturation, test devices were cleaned during the

[5] Animal 0103-05 was inadvertently not tested for pupil constriction.

[6] Animals 1006-02, 2121-02, and 0127-03 were tested in the same acoustic startle load cell positions on PND 23 and 60. Animal 2129-01 was tested in the same motor activity maze on PND 13 and 60. Animal 1130-08 was tested in the same motor activity maze on PND 21 and 60.

ensuing interval to reduce the residual scent from the other gender.

- Detailed Observational Battery (Set C)**:** On PND 4, 11, 21, 35 (±1 day), 45 (±1 day), and 60 (±2 days), approximately 20 offspring/sex/group (representing at least 20 litters per level) were examined outside the home cage for assessment, as appropriate for the developmental stage involved. This evaluation was performed according to the procedures described for maternal animals (see above), using standardized procedures. The only difference was that on PND 4 and PND 11, the pups were not likely to be evaluated in the open field, since this was routinely done only if the observer considered it necessary for evaluation and this was not normally the case in such young animals.
- Motor Activity Testing (Set A)**:** Motor activity was measured for approximately 20 rats/sex/dose (representing at least 20 litters per level) on PND 13, 17, 21, and 60 (±2 days)[7]. These animals were evaluated in the figure-eight maze for 60 min at each time point, using a computer-automated system (Universal Maze Monitoring System, Version 1.41, Columbus Instruments, Columbus, OH) and personal computer for automated data collection. The figure-eight maze was selected as an established and widely used automated activity device that can be used to detect both increases and decreases in activity. Each maze consists of a series of inter-connected alleys (approximately 10 x 10 cm in cross-section) converging on a central arena and covered by transparent acrylic plastic. Each maze has eight infrared emitter/detector pairs (three in each of the figure eight alleys and one in each of the blind alleys) to measure activity and an activity count was registered each time a beam was interrupted. The floor of each maze rests above absorbent paper, which was changed routinely at the end of each day. Broad-spectrum background noise [74 ± 2dB(A)] was provided throughout the test to minimize acoustical variations during testing. The uniformity of light intensity (100 ± 70 Lux) over each maze was verified daily. Motor and locomotor activities were examined as total activity counts (beam interruptions) for the 60-min session and as activity during each 10-min interval. Motor activity was measured as the number of beam interruptions that occur during the test session. Locomotor activity was measured by eliminating consecutive counts for a given beam. Thus, for locomotor activity, only one interruption of a given beam was counted until the rat relocates in the maze and interrupts a different beam. Habituation was evaluated as a decrement in activity over consecutive intervals of the test session.
- Auditory Startle Reflex Habituation (Set B)**:** Auditory startle reflex habituation testing was performed in approximately 20 rats/sex/dose (representing at least 20 litters per level) on PND 23 and 60 (±2 days), using an automated system. A personal computer was used to control the operation of an integrated startle response test system (Coulbourn Acoustic Startle, Version 3.210-00, Coulbourn Instruments, Allentown, PA) and for automated data collection. Groups of four animals (maximum) were tested simultaneously within each of two startle system enclosures. Each enclosure was ventilated, lined with sound-attenuating and vibration-absorbing material, and housed a speaker mounted in a central position within the ceiling of the enclosure to provide the eliciting stimulus (S2) - a 50-msec burst (0 msec rise/fall) of broad-

[7] Animal 1123-10 was determined to have been mis-sexed when animals were transferred to individual cages, so this animal's data was removed from analysis.

spectrum "white" noise [approximately 118 dB(lin)]. Each enclosure also housed four load cell/force transducer assemblies that are designed to measure the startle response. During the test session, animals were placed into individual restraining cages that are positioned on top of each load cell. The test session consisted of 50 trials that began following approximately a 5 min adaptation period at ambient noise levels. The rats were then presented with the startle-eliciting stimulus at 10-second intervals. The peak response amplitude was determined for each trial as described below. The average response amplitude and the magnitude of decrease (habituation) over blocks of ten trials were compared among the dosage groups. Data collection began with the presentation of S2 and continued thereafter for 200 msec. The analog signal for each response output (measured in mV) was digitized at one kHz (i.e., one sample/msec for 200 msec) and converted to grams using a previously determined calibration curve for each load cell. Peak response amplitude (g) and latency (msec) measurements were taken from each animal's individual response curve. Baseline was defined as the average force (g) exerted on the platform during the first 8 msec following the onset of S2, a time period that precedes response onset. This baseline value was taken to represent an approximate body weight measurement that was used to verify that the equipment used to measure the response amplitude was functioning properly. Response amplitude was defined as the maximum value of the average curve, minus the baseline (i.e., removing the animal's body weight from the measurement). Latency to peak was the time (msec) following the onset of S2 when the peak response amplitude occurs.

- Learning and Memory Testing (Set C): Learning and memory testing were performed in approximately 16 rats/sex/dietary level, representing at least 20 litters per level. The same set of animals were used for testing passive avoidance (on PND 23 and 30) and water maze [PND 60 (± 2 days) and again seven days later].

Postweaning - Passive Avoidance: Animals were tested for acquisition on PND 23 and for retention on PND 30. Testing was conducted using equipment and computer programs from Coulbourn Instruments (Graphic State Notation 2 Version 2.002-00, Allentown, PA). A personal computer was used to control the operation of the equipment and for automated data collection. Testing took place in individual isolation cubicles, each housing a single shuttle cage. Each isolation cubicle was lined with foam insulation to attenuate sound in the chamber and had a fan with a baffled air intake and exhaust system for ventilation. The shuttle cage consisted of a Plexiglas and stainless-steel rectangular chamber fitted with front-loading access. Each shuttle cage (15 inches wide x 7.25 inches deep) was separated into two compartments of equal size (approximately 7 x 7 inches) by a wall that supports a centrally-located sliding (guillotine-type) door. The two compartments were identical, except that the walls in one compartment were lined with black film (dark-side) and the walls in the other compartment were not lined and it was illuminated during the test with a high-intensity lamp. The lamp was switched on to illuminate the light compartment at the start of each trial and remained on until either the animal crossed to the dark compartment or the trial ended. The floor of the cage consisted of a grid of stainless-steel bars. The movement of the animal from the starting (light) side to the dark compartment was detected by a photocell system. A Coulbourn solid-state scanning shock generator was used to deliver a brief (0.5 second) pulse of mild (0.5 mA) distributed shock to the grid floor when the animal crossed to the dark compartment.

After adaptation, individual animals were placed individually into the “lighted” compartment of a

conditioning apparatus (the shuttle cage), facing toward the light. After approximately 60 seconds, the trial began with the light being illuminated to signal the beginning of the trial and the door separating the two compartments opening, so that each rat was provided access to the non-illuminated side of the cage. When the rat crossed into the dark compartment, the door automatically closed, the shock was delivered, and the light switched off - signaling the end of that trial. At that time, the animal was returned promptly to the holding cage to wait for the next trial. If the rat failed to cross within 180 seconds, it was returned to the holding cage and the latency assigned an arbitrary score of 180. This restriction dictates the use of nonparametric statistical analyses. The procedure was repeated until either the rat remained in the lighted compartment for 180 seconds on two consecutive trials or until 15 trials elapsed, whichever occurred first. Rats that failed to meet the criterion during the learning phase were assigned a value of 15 for the trials-to-criterion variable. The test was repeated seven days later[8]. For this second trial, rats were placed in the illuminated side of the apparatus, given a 60 second acclimation period, and the latency to enter the dark side recorded.
Animals that either failed to reach criterion performance within 15 trials or failed to cross during the first two trials during acquisition were excluded from the retention phase of the experiment[9]. The dependent measures were the number of trials-to-criterion, latency to cross on Trial 1 and Trial 2 (learning phase only), and the number of rats/group that failed to reach criterion within 15 trials (learning phase only).
Adult (PND 60) Offspring - Water Maze: Animals were tested on PND 60 (±2 days), and again seven days later. Only animals that demonstrated acquisition were tested for retention. The water in the M-maze was maintained at 22 ± 1 °C. The mazes were constructed of opaque Plexiglass, with corridors approximately five inches wide and walls approximately 16 inches high, with approximately 7.5 inches of water. This maze was selected as an established and widely-used device that can be used to measure associative learning and memory.
On each test trial, the rat was placed into the starting position at the base of the M-maze stem, located between the two lateral arms. On the first (learning) trial, the rat was required to enter both arms of the maze before being provided access to the exit ramp to escape the water and then removed from the maze. The initial arm chosen on this learning trial was designated the incorrect goal during the subsequent 15 trials (maximum). Rats that failed to make a correct goal choice within 60 seconds in any given trial were guided to the correct goal with the exit ramp and then removed from the water. Between trials, the animal was returned to a transport cage to wait for the next trial. The inter-trial interval was approximately 15 (±5) seconds. Each rat was required to reach a criterion of five consecutive errorless trials to terminate the test session. The maximum number of trials in any test session was fifteen. Latency (in seconds) to choose the correct goal or the maximum 60 second interval was recorded for each trial, as well as the number of errors (incorrect turns in the maze) during each trial.
Animals that satisfied the above criteria within the 15 trial limit were tested for retention seven days following acquisition (animals that failed to reach criterion during acquisition were excluded from the

[8] Animals 3220-08 and 3124-10 were not tested for retention despite meeting acquisition.
[9] Animals 1114-05 and 1116-12 were tested for retention despite failing to cross during acquisition. Data for these animals was excluded from analysis.

retention phase of the experiment)[10]. The correct goal and the criterion were the same for both sessions. Dosage groups were compared for the following dependent measures: measures for acquisition include the number of trials-to-criterion, the average number of errors (incorrect turns in the maze) for each trial and the latency (in seconds) to reach the correct goal on trial 2 (a measure of short-term retention). Measures for retention include the number of trials-to-criterion, the average number of errors for each trial, and the latency (in seconds) to reach the correct goal on trial 1 (a measure of long-term retention).

6) Ophthalmology

At approximately 50–60 days of age, ophthalmic exams were conducted using the males and females (a minimum of 10/sex/dietary level; representing at least 20 litters per level) that were selected for perfusion at study termination. The exam took place in a semi-darkened room. The pupillary reflex was tested using a penlight or transilluminator, with a mydriatic agent applied to each eye to dilate the pupil. The conjunctiva and cornea were examined either before or after pupillary dilatation. After mydriasis, the lens, vitreous humor, retina, choroid, and optic disc were examined using an indirect ophthalmoscope equipped with a condensing lens.

2. Postmortem observations:

Maternal Animals: Maternal animals were sacrificed by CO_2 asphyxiation on Day 21 of lactation following the weaning of their respective litters. The dams were discarded without postmortem examination. Females that were sperm positive and/or had an internal vaginal plug but did not deliver were sacrificed by CO_2 asphyxiation on or after GD 24 without necropsy examination.

Offspring:

Necropsy: The offspring selected for brain weight or neuropathological evaluation (Set D) were sacrificed on PND 21 or 75 (±5 days). In addition, randomly selected animals from Sets A–C that were used to measure fresh brain weight were sacrificed by CO_2 asphyxiation and underwent a necropsy examination. The necropsy involved an examination of all organs (including the brain), body cavities, cut surfaces, external orifices and surfaces, with all gross abnormalities recorded.

Perfusion: Animals selected for perfusion on PND 21 (from Set D) or at study termination (from Sets A–C) were deeply anesthetized using an intraperitoneal dose of pentobarbital (approximately 50 mg/kg) and then perfused via the left ventricle with a sodium nitrite (in phosphate buffer) flush followed by in situ fixation using universal fixative [4% (w/v) EM-grade formalin] in phosphate buffer. On PND 21, only the brain (with olfactory bulbs) was collected. At study termination, the brain and spinal cord, both eyes (with optic nerves) and selected (bilateral) peripheral nerves (sciatic, tibial, and sural), the gasserian ganglion, gastrocnemius muscle, both forelimbs, and physical identifier were collected. All tissues were post-fixed in 10% buffered formalin, with the exception of the eyes, which were post-fixed in Davidson's fixative. The brain was weighed upon removal from the skull, prior to placement into formalin, and the brain:body weight ratio calculated[11].

[10] One control female (0111-12) was inadvertently not tested for retention despite meeting acquisition.

[11] Only 9 females and 19 litters were represented at the control level for terminal body weights and brain:body weight ratio. A body weight was inadvertently not collected for animal 0129-07, so brain:body weight ratio could not be calculated.

Measurements: At necropsy and prior to placement in 10% formalin, a Vernier caliper was used to obtain two linear measurements (mm).

1. Anterior-to-posterior (AP) length of the cerebrum, extending from the anterior pole to the posterior pole, exclusive of the olfactory bulbs; and
2. Anterior-to-posterior (AP) length of the cerebellum, extending from the anterior edge of the cortex to the posterior pole.

These gross measurements were performed by an individual who was aware of dose group assignments.

Histology: The brain tissue from perfused animals and any gross lesions collected at necropsy were further processed for microscopic examination. After the gross measurements were taken, the brain was divided into eight coronal sections for microscopic examination. The eight brain sections were processed according to standard procedures for paraffin embedding, sectioned at approximately 5 µm, and examined after staining with hematoxylin and eosin (H&E). In addition, the brain sections (duplicate sections) reserved for morphometric measurements (levels 4, 5, and 7) were stained using luxol fast blue/cresyl violet. Additional tissues were collected for microscopic examination from animals that were perfused at study termination. This included three levels of the spinal cord (cervical, thoracic, and lumbar), the cauda equina, eyes, optic nerves, and gastrocnemius muscle embedded in paraffin and stained with H&E. Dorsal root ganglia (including dorsal and ventral root fibers) from the cervical and lumbar swellings and gasserian ganglia were embedded in glycol methacrylate (GMA). GMA-embedded tissues were sectioned at 2–3 µm and stained using a modified Lee's stain. Peripheral nerve tissues (sciatic, tibial, and sural nerves) were embedded in GMA resin and sectioned longitudinally. The sciatic nerve was also cut in cross section.

Brain tissue for morphometric analysis was processed to slides at all dose levels. For micropathology, tissues from low- and mid-dose animals remained in formalin as it was not necessary to examine these tissues.

The measurements for the brains for morphometric analysis were performed on the forebrain, midbrain, and cerebellum using the same structures and areas for both Day 21 and Day 70 rats.

Details of Measurement:

1. Forebrain between Bregma -0.12 and -0.36 mm (plate # 34–36)
2. Midbrain between Bregma -3.24 and -3.48 mm (plate # 60–62)
3. Cerebellum between Bregma -11.76 and -12.00 mm (plate # 131–133)

Initially, one section was used for morphometry and a second section was available as a back-up section. The acceptance/rejection criteria for selecting the slides for measurement are (1) the above mentioned brain areas and their corresponding Bregma numbers should correspond to the Bregma number/areas depicted in the atlas, "The Rat Brain in Stereotaxic Coordinates" by Paxinos and Watson (6th edition), and (2) no measurements were taken from sections that are damaged or not of an adequate transverse nature. As deemed necessary by the study pathologist, additional re-cuts were performed to achieve this.

The checked (x) tissues were evaluated for adult offspring.

	CENTRAL NERVOUS SYSTEM		PERIPHERAL NERVOUS SYSTEM
	BRAIN (8 levels, including)		PERIPHERAL NERVES
x	Forebrain	x	Sciatic
x	Center of cerebrum	x	Tibial
x	Midbrain	x	Sural
x	Cerebellum		
x	Pons		
x	Medulla oblongata		
	SPINAL CORD		OTHER
x	Cervical swelling	x	Lumbar dorsal root ganglion
x	Thoracic	x	Lumbar dorsal root fibers[1]
x	Lumbar swelling	x	Lumbar ventral root fibers[1]
	OTHER	x	Cervical dorsal root ganglion
x	Gasserian Ganglion	x	Cervical dorsal root fibers[1]
x	Optic nerves	x	Cervical ventral root fibers[1]
x	Eyes	x	Gastrocnemius muscle
x	Cauda equina		

[1] Dorsal and ventral root fibers were evaluated as they were generally included with the ganglion

Micropathology and Morphometry: The tissues from high-dose animals were examined, relative to those from the respective control group. Since there were no test article-related lesions evident, further analysis was not performed.

Selected brain regions underwent the following quantitative analysis, with the individual performing the measurements aware of dose assignments. Initially, five linear measurements were taken. Two of the five measurements involve gross measurements of the intact brain, as described above. The other three were taken from the histologic sections using software calibrated with an ocular micrometer. These three measurements are described as follows:

1. Frontal cortex thickness (Forebrain). This measurement was of the dorsal portion of the cerebral cortex within the coronal section passing through the region of the optic chiasm.
2. Hippocampal gyrus thickness (Midbrain). This measurement was of the full width of the hippocampal gyrus, from the ventral tail of the dentate gyrus to the overlying subcortical white matter. Measurements were taken from the hippocampus from both sides of this section, and the mean value recorded.
3. Cerebellum height (Cerebellum/Pons). This measurement extends from the roof of the fourth ventricle to the dorsal surface.

In addition to these measurements, all brain sections from these control and high-dose male and female offspring underwent an extensive micropathologic evaluation. Slides from mid- and low-dose animals were not analyzed as there were no findings at the high-dose that warrants examination of tissues from these dose groups.

D. Data Analysis

1. Statistical Analysis

Statistical evaluations were generally performed using software from INSTEM Computer Systems, TASC, or SAS. The level of significance was set at $p \leq 0.05$, with the exception of Bartlett's Test, which was tested at $p \leq 0.001$. In general, continuous data was initially assessed for equality of variance using Bartlett's Test. Group means with equal variances were analyzed further using an Analysis of Variance (ANOVA), followed by a Dunnett's Test if a significant F-value was determined in the ANOVA. In the event of unequal variances, these data were analyzed using nonparametric statistical procedures (Kruskal-Wallis ANOVA followed by the Mann-Whitney U Test for between-group comparisons). **Detailed observational battery,** continuous data were analyzed using an ANOVA, with post-hoc comparisons using Dunnett's Test. Categorical data were analyzed using General Linear Modeling and Categorical Modeling (CATMOD) Procedures, with post-hoc comparisons using Dunnett's Test and an Analysis of Contrasts, respectively. **Motor and locomotor activity** (total session activity and activity for each 10 min interval) was analyzed using ANOVA procedures. Session activity data for the four test occasions were analyzed using an ANOVA to determine whether there was a significant day by treatment interaction. Interval data was subjected to a Repeated-Measures ANOVA, using both test interval and test occasion as repeated measures, followed by an ANOVA to determine whether there was a significant treatment by interval interaction on each test occasion. **Auditory startle** response amplitude data (peak amplitude) for the two test occasions was first analyzed using an ANOVA procedure. If there is a significant group effect, Dunnett's Test was used to determine whether the treated group was significantly different from control. The response amplitude data for each block of ten trials (five blocks/test session) was subjected to a Repeated-Measures ANOVA using test block as the repeated measure. If there is a significant group by block interaction, the values for each block were subjected to analysis using Dunnett's Test to determine if the results for treated animals are significantly different from control. **Passive avoidance** data was analyzed as follows. Latency data was analyzed using a Wilcoxon Test for time to failure (i.e., time to cross). The number of trials-to-criterion was analyzed using Kruskal-Wallis and Wilcoxon Tests for the acquisition phase and Fisher's Exact Test for retention. The number of rats failing to meet the criterion level of performance in the learning (acquisition) phase were analyzed as incidence data. **Water maze** results were analyzed using parametric and non-parametric tests. Latency data were analyzed by a univariate ANOVA, with post-hoc analysis using Dunnett's Test. The number of trials-to-criterion and the number of errors were analyzed using Kruskal-Wallis and Wilcoxon Tests for the acquisition phase and Fisher's Exact Test for retention. The number of rats that failed to meet the criterion level of performance in the learning phase was analyzed as incidence data. **Pathology** data were screened for potential effects and then evaluated using the following approach. Statistical analyses used for organ weights, ophthalmology, gross pathology and neuropathology are depicted in Table 5.7.5-03.

Table 5.7.5-03: Statistical Analyses

DATA TYPE	DATA	STATISTICAL TESTS	COMPUTER
	Organ Weight	Bartlett's for Homogeneity, with ANOVA or Kruskal-Wallis (1)	DATATOX
	Gross Brain Measurements	Bartlett's for Homogeneity, with ANOVA or Kruskal-Wallis (1)	DATATOX
	Microscopic Brain Measurements	ANOVA and/or *t*-Test (2)	Microsoft Excel® Microsoft Office Excel® 2003 SP2, Version 11.6560.6568
FREQUENCY	Ophthalmology	Visually Screened (3)	Trend.exe
	Gross Pathology	Visually Screened (3)	DATATOX/Trend.exe
	Micropathology	Chi-Square Fisher's Exact Test	SAS

All statistical tests based significance level of $p \leq 0.05$, except for Bartlett's, which is based on $p \leq 0.001$
(1) ANOVA used if data were homogeneous; Kruskal-Wallis used if data were non-homogeneous
(2) A *t*-Test, 2-tailed, used for two-group comparisons; an ANOVA followed by a Dunnett's Test used for multiple-group comparisons
(3) If potential compound effects were suspected, then Chi-Square and one-tailed Fisher's Exact Tests were used.

2. Indices

***Reproductive Indices*:**

The following reproductive indices were calculated from breeding and parturition records of animals in the study:

Mating Index = (no. of inseminated females/no. of females co-housed with males) X 100
Fertility Index = (no. of pregnant females/no. of inseminated females) X 100

***Offspring Viability Indices*:**

The following viability (survival) indices were calculated from lactation records of litters in the study:

Live Birth Index = (no. of live pups born per litter/total no. of pups per litter) X 100
Viability Index = (no. of live pups on Day 4 pre-culling per litter/no. of live pups born per litter) X 100
Lactation Index = (no. of live pups on Day 21 per litter/no. of live pups on Day 4 post-culling per litter) X 100

II. Results and discussion

A. Parental Animals

1. Mortality and clinical and functional observations

No P-generation females were found dead during gestation or lactation. There were also no P-generation males found moribund or dead after initiation of the study (males did not receive the test substance).

There were no test substance-related clinical signs at any dietary level during gestation or lactation. During gestation, findings considered incidental and unrelated to the test substance included red vaginal discharge on GD 13 (1 mid-dose dam), fused digits (1 control dam), and hair loss (1 to 3 dams from all

dietary levels, including controls), which is a common finding associated with nest-building behavior in pregnant rats. During lactation, findings that are considered incidental and unrelated to the test substance included fused digits (1 control dam) and areas of hair loss (1 to 4 dams at all dietary levels, including control).

Table 5.7.5-04: Mortality and Clinical Signs in Maternal Animals

Observation	Dietary Level of BYI 02960			
	Control	120 ppm	500 ppm	1200 ppm
Gestation (Days 6–24)				
No. of Females Examined on GD 6	30	30	30	30
Red Vaginal Discharge	0	0	1	0
Hair Loss	1	1	2	3
Fused Digits	1	0	0	0
No. of Females Found Dead	0	0	0	0
Lactation (Days 0–21)				
No. of Females Examined on LD 0 [a]	27	29	29	30
Hair Loss	1	1	3	4
Fused Digits	1	0	0	0
No. of Females Found Dead	0	0	0	0

There were no test substance-related findings observed in the detailed observational battery in dams at any dietary level. Findings that are considered incidental and unrelated to the test substance included fused digits (1 control dam), lesion described as a scab (1 low-dose dam on LD 21), and areas of hair loss described as alopecia at various time points (1 to 3 dams at all dietary levels, including control). There was a statistical increase in the mean number of urination pools in the open field, compared to controls for mid- and high-dose females on LD 11 (3.7 and 2.6, respectively, versus 0.6 for controls). This difference from control is not thought to be test substance-related since the incidence was not dose related and occurred at only one time point (Table 5.7.5-05).

Table 5.7.5-05: Maternal Detailed Observations

Observation	Dietary Level of BYI 02960			
	Control	120 ppm	500 ppm	1200 ppm
Gestation Day 13				
No. of Animals Examined	30	30	30	30
Handling-Other; Alopecia				
Not Observed	29(97)	29(97)	28(93)	30(100)
Present	1(3)	1(3)	2(7)	0(0)
Gestation Day 20				
No. of Animals Examined	30	30	30	30
Handling-Other; Alopecia				
Not Observed	29(97)	29(97)	28(93)	27(90)
Present	1(3)	1(3)	2(7)	3(10)
Handling-Other; Fused Digits				
Not Observed	29(97)	30(100)	30(100)	30(100)
Present	1(3)	0(0)	0(0)	0(0)
Lactation Day 11				
No. of Animals Examined	10	10	10	10
Handling-Other; Alopecia				*
Not Observed	10(100)	10(100)	10(100)	7(70)
Present	0(0)	0(0)	0(0)	3(30)
Handling-Other; Fused Digits				
Not Observed	9(90)	10(100)	10(100)	10(100)
Present	1(10)	0(0)	0(0)	0(0)
Urination-Number of Pools; Mean± S.D.	0.6±0.8	2.0±1.8	*3.7±1.8	*2.6±2.3
Lactation Day 21				
No. of Animals Examined	10	10	10	10
Handling-Other; Alopecia				*
Not Observed	10(100)	10(100)	10(100)	7 (70)
Present	0(0)	0(0)	0(0)	3 (30)
Handling-Scab				
Not Observed	10(100)	9 (90)	10(100)	10(100)
Present	0(0)	1 (10)	0(0)	0(0)

Values represent the number of animals and % incidence (in parentheses) with or without observation.
*** Findings were statistically different from control, $p \leq 0.05$**
Severity: 0=Not Observed, 1=Slight, 2=Moderate to Severe

2. Body Weight and Food Consumption

During gestation, there was a statistical decrease (-7%), relative to controls, in mean body weight on GD 20 and a statistical decrease (-21%) in overall mean weight gain during gestation at the 1200 ppm dietary level. There were statistical decreases in body weights on LD 0 and 4 (-4% and -6%, respectively), relative to controls, at the 1200 ppm dietary level, and non-statistical decreases (-4% to -6%) that continued through termination on LD 21 at the 1200 ppm dietary level.There were no effects

on body weight or body weight gain during gestation or lactation at any other dietary level. There were no effects on food consumption during gestation or lactation at any dietary level. Effects on maternal body weight and food consumption are depicted below in Table 5.7.5-06.

Table 5.7.5.-06: Mean (±S.E.) Maternal Body Weight and Food Consumption

Observations/Study Week	Dietary Level of BYI 02960			
	Control	120 ppm	500 ppm	1200 ppm
Gestation				
Mean Body Weight (g) GD 0	206.4±2.18 (27)	208.0±2.54 (29)	209.6±2.36 (29)	209.8±2.59 (30)
Mean Body Weight (g) GD 6	229.1±2.71 (27)	230.8±2.96 (29)	231.2±3.46 (29)	233.1±2.72 (30)
Mean Body Weight (g) GD 13	254.8±3.74 (27)	256.0±3.58 (29)	256.0±3.27 (29)	245.3±2.92 (30)
Mean Body Weight (g) GD 20	320.9±4.09 (27)	314.7±5.14 (28)	318.7±4.26 (29)	299.9**±4.05 (29)
Mean Weight Gain (g) GD 0–20	114.5±2.89 (27)	107.5±3.38 (28)	109.1±2.59 (29)	90.3**±2.57 (29)
Mean Food Consumption (g/animal/day) GD 6–13	20.1±0.73 (27)	19.1±0.47 (28)	18.9±0.47 (29)	18.4±0.59 (30)
Mean Food Consumption (g/animal/day) GD 13–20	20.3±0.37 (27)	20.1±0.43 (27)	20.2±0.40 (29)	19.2±0.48 (29)
Lactation				
Mean body weight (g) LD 0	244.0±3.30 (27)	245.2±3.85 (29)	242.9±3.43 (29)	233.3*±2.76 (30)
Mean body weight (g) LD 4	262.0±3.19 (26)	256.7±4.48 (23)	256.1±3.16 (26)	247.2**±3.62 (25)
Mean body weight (g) LD 7	265.4±2.98 (23)	263.2±3.89 (23)	263.4±3.36 (23)	255.7±3.46 (23)
Mean body weight (g) LD 14	279.6±3.17 (23)	274.6±4.05 (23)	275.2±3.30 (23)	266.6±2.86 (23)
Mean body weight (g) LD 21	261.1±5.87 (23)	264.1±4.50 (23)	254.2±3.88 (23)	246.5±6.38 (23)
Mean food consumption (g/animal/day) LD 0–7	36.0±1.05 (23)	35.3±1.01 (23)	34.1±1.14 (23)	34.0±0.80 (23)
Mean food consumption (g/animal/day) LD 7–14	50.8±0.84 (23)	50.3±1.08 (23)	50.7±0.86 (23)	50.3±1.00 (23)
Mean food consumption (g/animal/day) LD 14–21	55.9±1.76 (23)	58.1±1.12 (23)	54.7±1.06 (23)	54.5±1.35 (23)

Values are mean ± S.E. (*n*). Means for gestation include only dams found sperm positive and with pups at termination of gestation.
* Statistically different from control, Dunnett's Test $p \leq 0.05$; ** Statistically different from control, Dunnett's Test $p \leq 0.01$

3. Test substance intake

The average daily intake of the active ingredient (A.I.) (mg A.I./kg body weight/day) was calculated using weekly body weight and food consumption data. The general relationship used for this

calculation was: [AI in feed (ppm)/1,000] x [feed consumed (g/kg body wt/day)] = mg AI/kg body wt/day. The average consumption of test substance for females that received diets containing nominal concentrations of 0, 120, 500, or 1200 ppm during gestation, with adjustments during lactation, are presented in Table 5.7.5.-07. Based on these results, the average daily intake of active ingredient during gestation and lactation was 0, 10.3, 42.4, and 101.7 mg/kg/day.

Table 5.7.5-07: Mean Maternal Test Substance Intake (mg/kg body weight/day)

Period	Dietary Level of BYI 02960		
	120 ppm	500 ppm	1200 ppm
Gestation			
Gestation Days 6–13	9.9±0.20 (28)	41.9±1.36 (29)	93.5±3.29 (30)
Gestation Days 13–20	9.4±0.14 (27)	40.0±0.56 (29)	92.7±2.42 (29)
Lactation			
Lactation Days 0–7	8.5±0.26 (23)	34.4±1.31 (23)	86.3±2.18 (23)
Lactation Days 7–14	11.2±0.18 (23)	47.0±0.68 (23)	115.7±2.00 (23)
Lactation Days 14–21	12.4±0.20 (23)	48.7±1.17 (23)	120.1±2.71 (23)

Values are mean ± S.E. (*n*). Dietary concentrations were reduced during Weeks 1–3 of lactation by 50%, based on estimated increases in feed consumption (g consumed/kg body wt./day) during lactation.
Nominal concentrations were calculated using the purity of 96.2%.

4. Reproductive performance

There were no effects on reproductive parameters at any dietary level (Table 5.7.5-08).

Table 5.7.5-08: Reproductive Performance

Observation	Dietary Level of BYI 02960			
	Control	120 ppm	500 ppm	1200 ppm
No. of Animals Co-housed [a]	30	30	30	30
No. of Animals Mated	30	30	30	30
Maternal Wastage				
No. of Dams not Pregnant	3	1	1	0
No. of Dams that Delivered Dead Pups	1	0	0	2
No. of Dams with Pre-Mature Delivery	0	0	0	0
Mating Index	100.0	100.0	100.0	100.0
Fertility Index (No. of pregnant females/No. of inseminated females X 100)	90.0	96.7	96.7	100.0
Gestation Length (days) [b]	21.7±0.10 [22.0] (21.0–22.0)	21.7±0.13 [22.0] (20.0–23.0)	21.8±0.13 [22.0] (21.0–23.0)	21.9±0.16 [22.0] (20.0–23.0)

[a] **Number of animals assigned to each dietary level.**

[b] **Values are mean ± S.E., [median], and (range).**

Values were not statistically different from control, $p \leq 0.05$ or $p \leq 0.01$

5. Maternal postmortem results – not applicable to the present study.

B. Offspring

1. Viability and clinical signs

Litter parameters and pup viability were not affected by the test substance at any dietary level (Table 5.7.5-09)..

Table 5.7.5-09: Litter Size and Viability

Observation	Dietary Level of BYI 02960			
	Control	120 ppm	500 ppm	1200 ppm
No. of Litters	23	23	23	23
Total No. of Pups Born	269	265	275	255
Total No. of Pups Missing	0	0	0	1
Litters with Pups Missing	0	0	0	1
Total No. of Pups Found Dead	2	2	0	0
Litters with Pups Found Dead	2	2	0	0
Total No. of Pups Cannibalized	0	0	0	0
Litter with Pups Cannibalized	0	0	0	0
Litter Size	11.7±0.34 [12.0] (8.0–15.0)	11.5±0.39 [12.0] (8.0–14.0)	12.0±0.36 [12.0] (8.0–15.0)	11.1±0.38 [11.0] (7.0–14.0)
Stillborn Pups				
Number	1	0	0	2
%	0.4	0.0	0.0	0.8
Mean±S.E.	0.0±0.04	0.0±0.00	0.0±0.00	0.1±0.06
[Median]	[0.0]	[0.0]	[0.0]	[0.0]
(Range)	(0.0–1.0)	(0.0–0.0)	(0.0–0.0)	(0.0–1.0)
Mean No. of Viable Pups				
Birth	12	12	12	11
Day 4 (Pre-cull) [a]	12	11	12	11
Day 4 (Post-cull) [b]	8	8	8	8
Day 21	8	8	8	8
Live Birth Index [c]	99.6±0.43 [100.0] (90-100)	100.0±0.00 [100.0] (100-100)	100.0±0.00 [100.0] (100-100)	99.3±0.51 [100.0] (91–100)
Viability Index [c]	99.3±0.51 [100.0] (91–100)	99.1±0.64 [100.0] (89–100)	100.0±0.00 [100.0] (100–100)	100.0±0.00 [100.0] (100–100)
Lactation Index [c]	100.0±0.00 [100.0] (100–100)	100.0±0.00 [100.0] (100–100)	100.0±0.00 [100.0] (100–100)	99.5±0.54 [100.0] (88–100)

[a] Before standardization (culling) [b] After standardization (culling) [c] Values are mean ± S.E., [median], (range)
Values were not statistically different from control, $p \leq 0.05$ or $p \leq 0.01$

There were no test substance-related clinical signs during lactation or postweaning in males or females at any dietary level.

2. Body weight

Preweaning (Table 5.7.5-10):

Body weight was non-statistically decreased in high-dose males and females on PND 17 and 21 (-4% to -5%). Although these differences from control are small and not statistically significant, they are attributed to the test substance because of the relationship with dose (both genders and at multiple time points). These lower body weights were comparable to the 4–6% lower body weight for the high-dose

dams throughout lactation. Body weight was not affected by the test substance in either sex at any other dietary levels.

Body weight gain for PND 4–17 was statistically decreased in high-dose males (-7%) and males and females combined (-6%). Body weight gain for the shorter period of PND 11–17 was statistically decreased in high-dose males (-10%), females (-7%), and males and females combined (-8%). There were similar non-statistical trends in body weight gain for PND 4–17 in high-dose females (-6%), for PND 4–21 and PND 11–21 in both sexes, and in both sexes combined (-5% to -6%). These differences from control are attributed to the test substance. Body weight gain was not affected by the test substance in either sex at the 120 and 500 ppm dietary levels.

Table 5.7.5-10: Mean (± S.E.) Preweaning Pup Body Weights and Body Weight Change (g)

Postnatal Day	Dietary Level of BYI 02960							
	Control	120 ppm	500 ppm	1200 ppm	Control	120 ppm	500 ppm	1200 ppm
	Males				Females			
0	5.9±0.13 (23)	5.9±0.10 (23)	6.0±0.10 (23)	6.0±0.10 (23)	5.6±0.11 (23)	5.7±0.10 (23)	5.7±0.08 (23)	5.7±0.11 (23)
4 [a]	9.9±0.25 (23)	9.8±0.20 (23)	9.9±0.24 (23)	10.0±0.26 (23)	9.5±0.23 (23)	9.5±0.20 (23)	9.4±0.21 (23)	9.5±0.28 (23)
4 [b]	10.0±0.25 (23)	9.8±0.20 (23)	9.9±0.24 (23)	9.9±0.26 (23)	9.5±0.24 (23)	9.5±0.20 (23)	9.4±0.21 (23)	9.4±0.28 (23)
11	25.4±0.48 (23)	24.9±0.46 (23)	25.7±0.44 (23)	24.5±0.49 (23)	24.5±0.44 (23)	24.3±0.46 (23)	24.6±0.42 (23)	23.7±0.47 (23)
17	38.9±0.65 (23)	37.9±0.55 (23)	39.2±0.56 (23)	36.8±0.60 (23)	37.5±0.56 (23)	37.0±0.59 (23)	37.6±0.47 (23)	35.8±0.54 (23)
21	47.9±1.04 (23)	48.4±0.85 (23)	47.6±0.66 (23)	45.6±1.02 (23)	46.3±1.01 (23)	47.2±0.96 (23)	45.4±0.65 (23)	44.4±0.83 (23)
	Body Weight Change							
	Males				**Females**			
4–17	28.9±0.56 (23)	28.1±0.45 (23)	29.3±0.41 (23)	26.9±0.51* (23)	27.9±0.50 (23)	27.5±0.48 (23)	28.2±0.33 (23)	26.3±0.43 (23)
4–21	37.9±0.88 (23)	38.6±0.73 (23)	37.7±0.57 (23)	35.7±0.87 (23)	36.8±0.88 (23)	37.7±0.82 (23)	36.0±0.56 (23)	34.9±0.68 (23)
11–17	13.6±0.31 (23)	13.0±0.20 (23)	13.5±0.23 (23)	12.3±0.25** (23)	13.0±0.29 (23)	12.7±0.22 (23)	13.0±0.21 (23)	12.1±0.18* (23)
11–21	22.5±0.75 (23)	23.5±0.47 (23)	22.0±0.52 (23)	21.1±0.68 (23)	21.9±0.77 (23)	22.9±0.56 (23)	20.8±0.51 (23)	20.7±0.58 (23)
	Body Weight Change, Males and Females Combined							
	Control		**120 ppm**		**500 ppm**		**1200 ppm**	
4–17	28.4±0.52 (23)		27.8±0.45 (23)		28.7±0.35 (23)		26.6±0.45* (23)	
4–21	37.4±0.87 (23)		38.2±0.77 (23)		36.9±0.54 (23)		35.3±0.76 (23)	
11–17	13.3±0.29 (23)		12.8±0.20 (23)		13.3±0.20 (23)		12.2±0.19** (23)	
11–21	22.2±0.75 (23)		23.2±0.50 (23)		21.4±0.49 (23)		20.9±0.61 (23)	

Values are mean ± S.E. (*n*)

[a] Before standardization (culling); * Values were statistically different from control, $p \leq 0.05$

[b] After standardization (culling); ** Values were statistically different from control, $p \leq 0.01$

***Postweaning* (Table 5.7.5-11):**

There was no difference in body weight after weaning for males and females at any dietary level. For clarification, group average body weight data provided in the summary table are based on the mean for each litter, not for each individual pup.

Table 5.7.5-11: Mean (± S.D.) Postweaning Pup Body Weights (g)

Postnatal Day [a]	Dietary Level of BYI 02960							
	Control	120 ppm	500 ppm	1200 ppm	Control	120 ppm	500 ppm	1200 ppm
	Males				Females			
28	82.5±9.5 (23)	81.6±7.7 (23)	81.9±7.7 (23)	78.6±9.2 (23)	78.5±8.2 (23)	80.2±7.2 (23)	78.8±6.8 (23)	77.5±7.4 (23)
35	129.9±11.9 (23)	129.2±11.2 (23)	129.1±11.8 (23)	125.2±12.1 (23)	114.1±10.2 (23)	117.4±8.4 (23)	116.1±6.5 (23)	114.2±8.8 (23)
42	177.3±14.7 (23)	176.9±12.3 (23)	177.5±13.1 (23)	171.2±13.8 (23)	138.3±11.7 (23)	141.3±8.4 (23)	140.8±7.1 (23)	138.6±9.9 (23)
49	220.9±17.8 (23)	218.7±14.6 (23)	222.3±16.3 (23)	214.8±16.7 (23)	158.5±10.6 (23)	158.8±8.9 (23)	156.4±8.6 (23)	155.6±11.7 (23)
56	267.9±21.5 (23)	266.0±16.1 (23)	269.8±19.4 (23)	260.9±19.5 (23)	176.8±11.7 (23)	176.4±9.9 (23)	176.6±8.6 (23)	176.0±11.6 (23)
63	305.3±23.6 (23)	303.2±17.4 (23)	303.7±23.4 (23)	292.5±23.7 (23)	190.7±11.5 (23)	188.7±9.4 (23)	189.6±9.5 (23)	189.9±12.7 (23)
70	333.6±25.8 (23)	330.2±17.7 (23)	332.7±24.1 (23)	322.0±24.3 (23)	204.5±12.8 (23)	200.9±10.1 (23)	202.4±9.9 (23)	201.3±13.6 (23)

Values are mean ± S.D. (*n*); Values were not statistically different from control, $p \leq 0.05$

[a] Actual days of measurement occurred during the week of PND 28, 35, 42, 49, 56, 63, and 70.

3. Developmental Landmarks

The ages for onset of balanopreputial separation, vaginal patency, and surface righting were not affected by the test substance at any dietary level.

Pupil constriction in response to a penlight was apparent in all control and treated pups on PND 21. Therefore, there was no indication of a test substance-related effect at any dietary level.

The data are presented in Table 5.7.5-12.

Table 5.7.5-12. Mean (± S.E.) Age of Sexual Maturation and Surface Righting (days)

Parameter	Dietary Level of BYI 02960			
	Control	120 ppm	500 ppm	1200 ppm
Number of Litters (M/F)	23/23	23/23	23/23	23/23
Balanopreputial Separation % Pups Reaching Criteria	42.7±0.35 (100)	44.2±0.58 (100)	42.9±0.33 (100)	43.2±0.29 (100)
Vaginal Opening % Pups Reaching Criteria	32.3±0.51 (100)	33.9±0.85 (100)	33.4±0.65 (99)	32.3±0.44 (100)
Surface Righting % Pups Reaching Criteria	5.2±0.17 (100)	5.2±0.14 (100)	5.2±0.17 (100)	5.1±0.21 (100)
Pupil Constriction % Pups Reaching Criteria	21.0±0.00 (100)	21.0±0.00 (100)	21.0±0.00 (100)	21.0±0.00 (100)

Values are mean ± S.E.

Values were not statistically different from control, $p \leq 0.05$

4. Behavioral assessments:

a. Detailed observational battery:

There were no test substance-related findings in males or females at any dietary level. There was one incidence of respiratory abnormality (described as rapid, shallow breathing) observed in one control female on PND 45. This is not considered noteworthy since it occurred at only one time point in a control animal, was not observed during routine clinical observations, and was not seen in any other animals.

Incidental findings in males on various test days were exophthalmia (one mid-dose on PND 35), dermal lesion described as a scab (one control on PND 45, two controls and one low-dose on PND 60), and alopecia (one high-dose on PND 60). Incidental findings in females on various test days included red lacrimal stain (one low-dose on PND 35 and 60), red lacrimation (one low-dose on PND 60), and a dermal lesion described as a scab (one mid-dose on PND 60).

b. Motor activity:

Age-related changes in the levels of motor and locomotor activity were evident in control males and females. The greatest difference in activity for the overall 60 min test session was apparent as very low levels of locomotor activity in the youngest animals (PND 13), compared to subsequent test occasions. This outcome is consistent with their relatively undeveloped ambulatory skills and sensory function (e.g., eyelids closed), which is characteristic of animals at this age. This was followed by a progressive increase in levels of motor and locomotor activity with age. Gender-related differences in activity were apparent on PND 60 only, with modestly higher levels of motor and locomotor activity for females, compared with males. These comparisons (within the control group) describing performance by age and gender were not subjected to statistical analysis.

A non-statistical increase in both motor and locomotor activity was measured in high-dose males on PND 13. These differences from control are considered test substance-related, but are also partially attributed to a low mean average activity for controls (55 and 5, for motor and locomotor, respectively), which was below the range for historical controls (59–72 and 6–11, respectively) in the last five studies conducted at this laboratory.

An increase in motor activity was measured in low- and high-dose males on PND 17. These differences from control are not attributed to the test substance, since there was no dose-related pattern and the increases were not observed in mid-dose males or in females. Additionally, the increases were not observed on any subsequent test occasions, and means for all groups were within the range of historical controls.

An increase in locomotor activity was measured in low-, mid-, and high-dose males on PND 17. These differences from control are not attributed to the test substance, since there was no dose-related trend, increases were not observed in females and the means were within the range of historical controls. Lastly, very slight increases in locomotor activity were measured in low-dose males on PND 21 and high-dose females on PND 60. These are not considered test substance-related since motor activity for these same animals was generally comparable to controls, other dose levels were not similarly affected, and the differences were seen in only one sex.

The data are presented in Table 5.7.5-13.

Table 5.7.5-13: Mean (±S.D.) Motor Activity Data (total activity counts for session)

Test Day	Dietary Level of BYI 02960			
	Control	120 ppm	500 ppm	1200 ppm
Males				
PND 13	55±55 (20)	66±82 (20)	63±66 (20)	114±130 (20)
PND 17	146±117 (20)	260±104 (20)	173±114 (20)	221±144 (20)
PND 21	307±119 (20)	367±132 (20)	331±149 (20)	367±123 (20)
PND 60	503±142 (20)	518±108 (20)	505±121 (20)	529±111 (20)
Females				
PND 13	74±141 (20)	88±81 (19)	68±60 (20)	62±40 (20)
PND 17	201±152 (20)	149±112 (19)	243±121 (20)	227±200 (20)
PND 21	303±88 (20)	333±140 (19)	308±132 (20)	322±119 (20)
PND 60	656±143 (20)	666±233 (19)	624±202 (20)	790±232 (20)

Values are mean ± S.D. (n) Values were not statistically different from control, $p \leq 0.05$

Motor and locomotor activity data were also subjected to analysis at each 10-min interval of the 60-min test session. Evaluation of the progressive decrease in activity over the course of a test session provides a measure of habituation. For motor activity, habituation was evident in control males and females at all ages. For locomotor activity, habituation was apparent in controls at all ages except PND 13, when locomotor activity was quite low for all six intervals of the test session.

An analysis of the data by test interval indicated a sustained higher level of both motor and locomotor activity in high-dose males on PND 13. This difference from controls is considered test substance-related and accounts for the overall increase in motor activity for these animals on this test day, noted above. There were no other test substance-related effects in males, or in females at any other dietary level.

c. Auditory startle habituation

The amplitude of the startle response increased with age in both sexes. This reflects a true age-related increase in the force of the response, since body weight is not included in the measure of response amplitude (see Methods). The average response amplitude on PND 23 and 60 (± 2 days) was 41 g and 211 g, respectively, for control males, and 35 g and 78 g, respectively, for control females. These results also reflect a gender difference in response amplitude on PND 60. Habituation was apparent in control males and females as a decrease in response amplitude over the course of the test session. These comparisons (within the control group) to describe performance by age were not subjected to statistical analysis.

Startle amplitude for all 50 trials was statistically increased two-fold in high-dose females (156 g), compared to controls (78 g) on PND 60. This was reflected as a non-statistical increase in response

amplitude for the first block of trials, and a statistical increase in blocks 2–5, relative to control. This finding is considered to be related to the test substance. There was a non-statistical trend involving a slight increase in average startle amplitude in all five blocks of trials, in females at the low- and mid-dose, relative to controls, on PND 60. This difference from control is not thought to be test substance-related since the slight increase was similar in both dose levels with a 4-fold increase in dose and the mean average for startle response amplitude in control animals (78 g) is below the range for historical controls (87–116 g) in the last five studies conducted in this laboratory.

There were no differences in startle amplitude in male and female weanlings or in adult males at any dietary level age. Startle latency and habituation were not affected by the test substance in males or females at any dietary level, on any test occasion.

The data for auditory startle peak amplitude are presented in Table 5.7.5-14.

Table 5.7.5-14. Auditory Startle Reflex Peak Amplitude Data (g, mean ± S.D.)

Block		Dietary Level of BYI 02960			
		Control	120 ppm	500 ppm	1200 ppm
Males					
PND 23	Block 1	44±18	48±13	43±18	46±22
	Block 2	43±16	50±13	42±23	41±15
	Block 3	39±16	48±15	40±23	42±14
	Block 4	40±17	48±14	38±17	41±15
	Block 5	37±16	45±15	37±14	39±12
	Avg. For Total Session	41±15	48±12	40±18	41±13
	No. Of Animals	20	20	20	20
	Body Weight	60	60	60	57
PND 60	Block 1	270±115	301±159	241±151	324±162
	Block 2	255±161	299±190	203±141	289±215
	Block 3	200±140	245±157	175±136	214±183
	Block 4	187±149	214±140	152±130	177±165
	Block 5	148±92	187±131	134±146	144±108
	Avg. For Total Session	211±115	249±145	181±127	230±153
	No. Of Animals	20	20	20	20
	Body Weight	292	291	280	285
Females					
PND 23	Block 1	39±22	38±16	35±21	45±17
	Block 2	39±22	36±16	36±18	43±17
	Block 3	36±19	37±18	34±18	43±17
	Block 4	32±14	37±17	31±18	39±16
	Block 5	29±15	34±15	28±14	36±16
	Avg. For Total Session	35±17	36±14	33±17	41±15
	No. Of Animals	20	20	20	20
	Body Weight	57	58	56	57
PND 60	Block 1	102±74	133±75	140±91	186±129
	Block 2	94±82	121±94	127±84	181±126 ***
	Block 3	71±73	108±86	113±76	160±116 ***
	Block 4	68±54	84±58	96±65	132±87 ***
	Block 5	58±33	74±46	76±50	122±83 ***
	Avg. For Total Session	78±57	104±65	110±68	156±102 ***
	No. Of Animals	20	20	20	20
	Body Weight	185	180	184	174

Values are mean ± S.D. ***** Statistically different from control, ANOVA $p \leq 0.05$.**

d. Learning and memory testing:

Postweaning - Passive avoidance:

Acquisition and retention were clearly evident in control males and females. On the first test occasion, acquisition was evident in males and females as a marked increase in the latency to cross for the second trial (an average 172.1 and 166.0 seconds, respectively), compared to the first trial (an average 37.4 and 44.6 seconds, respectively). Thus, acquisition of the avoidance response (a failure to cross within the

180 seconds time limit for a trial) was quickly attained in control males and females. On the second test occasion, which occurred one week after the first, retention was evident in control males and females as a protracted delay to cross within the 180 seconds time limit of the first trial (an average 169.0 and 157.2 seconds, respectively), compared to the first trial on the first test day (an average 37.4 and 44.6 seconds, respectively). Retention was also evident in control males and females by a reduced average number of trials-to-criterion on the second test occasion (2.3 and 2.4 trials, respectively), compared to the first (3.1 and 3.3 trials, respectively). These comparisons (within the control group) to verify changes in performance with experience (acquisition/learning and retention/memory) were not subjected to statistical analysis.

As per standard procedure, animals that either failed to satisfy the criteria used to establish acquisition or that failed to cross during the first two trials (and therefore never received the conditioning stimulus) were not tested for retention. There was one low-dose male and female each, and one control female that did not cross during the learning phase.

There were no differences in acquisition or retention attributable to exposure to the test substance in either sex at any dietary level. The data are presented below in Table 5.7.5-15.

Table 5.7.5-15: Passive Avoidance Performance at PND 23 and 30 (mean ± S.D.)

Session/Parameter		Dietary Level of BYI 02960			
		Control	120 ppm	500 ppm	1200 ppm
Males					
Session 1 (Learning Phase)	Number of Animals Tested	16	16	16	16
	Number of Animals Included in Analysis	16	16	16	16
	Trials to criterion	3.1±0.3	3.3±0.9	3.9±3.0	3.1±0.3
	Latency trial 1 (seconds)	37.4±28.8	32.5±44.6	21.2±21.2	33.0±27.6
	Latency trial 2 (seconds)	172.1±31.8	163.9±44.5	154.4±55.0	174.7±21.3
	Failed to Meet Criterion	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Failed to Cross During Learning Phase	0 (0%)	1 (6%)	0 (0%)	0 (0%)
Session 2 (Retention Phase)	Number of Animals Tested	16	16	16	16
	Number of Animals Included in Analysis	16	15	16	16
	Trials to criterion	2.3±0.6	2.7±1.0	2.4±0.8	2.0±0.0
	Latency trial 1 (seconds)	169.0±30.5	156.6±61.9	149.6±59.9	180.0±0.0
	Latency trial 2 (seconds)	178.0±7.9	160.7±30.0	180.0±0.0	180.0±0.0
Females					
Session 1 (Learning Phase)	Number of Animals Tested	16	16	16	16
	Number of Animals Included in Analysis	16	16	16	16
	Trials to criterion	3.3±0.8	3.0±0.4	3.1±0.3	3.0±0.0
	Latency trial 1 (seconds)	44.6±41.9	53.3±57.4	35.5±34.0	22.7±20.5
	Latency trial 2 (seconds)	166.0±41.0	178.4±6.4	166.1±37.9	180.0±0.0
	Failed to Meet Criterion	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Failed to Cross During Learning Phase	0 (0%)	1 (6%)	0 (0%)	0 (0%)
Session 2 (Retention Phase)	Number of Animals Tested	16	16	16	14
	Number of Animals Included in Analysis	16	15	16	14
	Trials to criterion	2.4±0.6	2.6±0.9	2.3±0.6	2.2±0.6
	Latency trial 1 (seconds)	157.2±53.0	160.2±42.2	161.2±40.8	157.7±56.6
	Latency trial 2 (seconds)	179.1±3.5	179.6±1.4	173.0±28.2	174.4±20.8

Trials to Criterion = Mean No. Trials per Group ± S.D.
Latency to Trial 1 = Mean Session 1 duration (seconds) per Group ± S.D.
Latency to Trial 2 = Mean Session 2 duration (seconds) per Group ± S.D.
Failed to Meet Criterion = Number of animals that received the shock but did not demonstrate acquisition.
Failed to Cross = Number of animals that never received the shock.
Values were not statistically different from control, $p \leq 0.05$.

Adult Offspring – Water maze:

Acquisition and retention were evident in control males and females. On the first test occasion, acquisition was evident in controls as a progressive decrease in the average time to escape (to reach the exit ramp) over successive trials. For males the average trial duration (time to escape) decreased from

the first trial (an average 18.3 seconds) to the second trial (an average 16.6 seconds). By comparison for females, the average trial duration (time to escape) decreased from the first trial (an average 15.3 seconds) to the second trial (an average 13.1 seconds). On the second test occasion, the number of trials to criterion decreased, relative to performance for acquisition, in males (5.1 versus 7.3 for acquisition) and in females (6.2 versus 8.0 for acquisition). Additionally, retention was evident as a shorter trial duration for the first trial, compared to the first trial of acquisition for males (4.7 versus 18.3 seconds for acquisition) and females (7.3 versus 15.3 seconds for acquisition).

As per standard procedure, animals that failed to demonstrate acquisition were not tested for retention. There were three males (one control, one low-, and one high-dose) and four females (one control and three low-dose) that failed to demonstrate acquisition. Remaining males and females at all dietary levels demonstrated acquisition.

There were no differences in acquisition or retention attributable to exposure to the test substance in either sex at any dietary level. The data are shown below in Table 5.7.5-16.

Table 5.7.5-16: Water Maze Performance on PND 60 (± 2 days) and Seven Days Later (mean ± S.D.)

Session/Parameter		Dietary Level of BYI 02960			
		Control	120 ppm	500 ppm	1200 ppm
Males					
Session 1 (Learning Phase)	Number of Animals	16	16	16	16
	Trials to Criterion (Mean±S.D.)	7.3±2.7	8.3±2.9	6.4±1.5	7.6±3.0
	Trial 1 - Errors (Mean±S.D.)	0.6±0.7	0.9±1.3	0.5±0.7	0.5±0.6
	Trial 1 - Duration (seconds) (Mean±S.D.)	18.3±16.7	19.5±13.8	17.7±16.2	16.7±8.4
	Trial 2 - Errors (Mean±S.D.)	0.7±0.9	0.8±0.7	0.5±0.5	0.6±0.9
	Trial 2 - Duration (seconds) (Mean±S.D.)	16.6±18.4	19.6±12.3	17.2±13.6	12.3±9.8
	Failed to Meet Criterion	1 (6%)	1 (6%)	0 (0%)	1 (6%)
Session 2 (Retention Phase)	Number of Animals	15	15	16	15
	Trials to Criterion (Mean±S.D.)	5.1±0.3	6.0±1.8	5.3±0.6	5.4±0.6
	Trial 1 - Errors (Mean±S.D.)	0.1±0.3	0.3±0.6	0.3±0.6	0.7±1.6
	Trial 1 - Duration (seconds) (Mean±S.D.)	4.7±3.0	6.2±5.1	7.1±5.9	8.7±9.9
	Trial 2 - Errors (Mean±S.D.)	0.0±0.0	0.3±0.5	0.1±0.5	0.1±0.5
	Trial 2 - Duration (seconds) (Mean±S.D.)	2.5±1.0	5.7±4.9	3.6±4.7	3.0±3.7
Females					
Session 1 (Learning Phase)	Number of Animals	16	16	16	16
	Trials to Criterion (Mean±S.D.)	8.0±2.9	7.9±3.9	7.6±2.3	7.1±2.0
	Trial 1 - Errors (Mean±S.D.)	0.4±0.5	1.0±1.8	0.7±0.9	0.5±0.6
	Trial 1 - Duration (seconds) (Mean±S.D.)	15.3±9.2	18.6±14.0	16.3±12.8	14.6±7.6
	Trial 2 - Errors (Mean±S.D.)	0.5±0.7	0.3±0.6	0.8±1.0	0.6±.0.7
	Trial 2 - Duration (seconds) (Mean±S.D.)	13.1±8.2	11.3±15.1	16.0±14.9	12.8±4.4
	Failed to Meet Criterion	1 (6%)	3 (19%)	0 (0%)	0 (0%)
Session 2 (Retention Phase)	Number of Animals	14	13	16	16
	Trials to Criterion (Mean±S.D.)	6.2±1.4	6.9±2.9	5.7±1.7	5.9±1.3
	Trial 1 - Errors (Mean±S.D.)	0.3±0.5	0.5±1.0	0.3±0.8	0.6±1.2
	Trial 1 - Duration (seconds) (Mean±S.D.)	7.3±4.5	10.2±8.7	7.2±7.7	8.4±8.0
	Trial 2 - Errors (Mean±S.D.)	0.3±0.6	0.0±0.0	0.1±0.3	0.3±0.8
	Trial 2 - Duration (seconds) (Mean±S.D.)	5.4±4.3	3.8±2.2	5.1±4.8	5.1±4.4

Values for rats who failed to learn during Session 1 were not included in means for Session 2.
Values were not statistically different from control, $p \leq 0.05$.

e. Ophthalmology

At approximately 50–60 days of age, ophthalmic exams were conducted using the males and females that were selected for perfusion at study termination.
There were no test substance-related lesions in males or females at any dietary level.f. Postmortem results:
1. Gross Pathology:
There were no test substance-related gross pathology findings noted in PND 21 pups and PND 75 (± 5 days) rats at any dietary level.
There were no test substance-related gross pathology findings in PND 75 (± 5 days) nonperfused rats at any dietary level.
2. Terminal Body Weight:
PND 21 Perfused Pups: Test substance-related mean terminal body weight changes for PND 21 pups were noted in males at 1200 ppm dietary level (non-statistically decreased, 8%, as compared to controls) and in females at 120, 500, and 1200 ppm dietary levels (non-statistically decreased, -5, -7%, and -8%, respectively, as compared to controls).
Terminal body weights are collected once at termination from a sub-group of animals in each dose level (i.e., those animals scheduled for necropsy, 10/sex/dietary level). In-life body weights are collected from all animals at selected time points throughout the in-life phase of the study (e.g., mean litter weight is calculated using 23 litters/level). Although the sub-group of animals weighed at termination did indicate an apparent effect on body weight, in-life data clearly indicates no effect on body weight (compared to controls) in females from the 120 and 500 ppm dietary levels (see Table 5.7.5-10).
PND 75 (± 5 days) Perfused Rats: There was no test substance-related terminal body weight change noted at any dietary level.
PND 75 (± 5 days) Non-perfused Rats: There was a slight trend of decline in terminal body weights at 1200 ppm males (-5%, as compared to controls). There was a decrease (-5%) in terminal body weights observed in 120 and 1200 ppm females; however, these changes were not considered test substance-related due to lack of dietary relationship or the changes were small.
3. Brain Weights:
PND 21 Pup Brain Fixed Weight: There were no test substance-related changes in mean absolute or relative brain weights for Day 21 pups at any of the dietary level.
PND 75 (± 5 days) Rat Brain Fixed Weight: There were no test substance-related differences from controls in absolute or relative mean brain fixed weights for PND 75
(± 5 days) rats at any dietary level.
PND 75 (± 5 days) Rat Fresh Brain Weight: There was a decrease (-5%) in fresh brain weights at 1200 ppm males as compared to controls; however, this change was not considered directly related to test substance administration since it was related to the decrease in terminal body weights. Mean terminal body weight and brain weight data are also presented in Table 5.7.5-17.

Table 5.7.5-17: Mean (± S.D.) Brain Weight Data

Parameter	Dietary Level of BYI 02960			
	Control	120 ppm	500 ppm	1200 ppm
Males				
PND 21(Perfused)				
Terminal Body Weight (g)	49.0±5.0 (10)	49.6±5.5 (10)	50.6±3.6 (10)	45.2±4.0 (10)
Brain, Fixed (g)	1.415±0.059 (10)	1.401±0.054 (10)	1.404±0.066 (10)	1.386±0.056 (10)
Brain, Fixed/Body Weight (%)	2.921±0.359 (10)	2.849±0.274 (10)	2.786±0.177 (10)	3.083±0.228 (10)
PND 75 (±5) (Perfused)				
Terminal Body Weight (g)	335.4±28.6 (10)	339.8±14.0 (10)	339.8±40.9 (10)	332.6±28.1 (10)
Brain, Fixed (g)	1.836±0.107 (10)	1.885±0.080 (10)	1.858±0.098 (10)	1.865±0.091 (10)
Brain, Fixed/Body Weight (%)	0.549±0.031 (10)	0.555±0.028 (10)	0.554±0.069 (10)	0.545±0.040 (10)
PND 75 (±5) (Non-Perfused)				
Terminal Body Weight (g)	334.6±24.2 (10)	334.7±24.0 (10)	341.7±30.5 (10)	318.7±35.0 (10)
Brain, Fresh (g)	1.956±0.090 (10)	1.985±0.034 (10)	1.949±0.070 (10)	1.868*±0.085 (10)
Brain, Fresh/Body Weight (%)	0.587±0.046 (10)	0.596±0.041 (10)	0.574±0.046 (10)	0.591±0.049 (10)
Females				
PND 21(Perfused)				
Terminal Body Weight (g)	48.9±5.2 (10)	46.6±5.1 (10)	45.3±3.4 (10)	44.9±2.8 (10)
Brain, Fixed (g)	1.378±0.081 (10)	1.349±0.080 (10)	1.341±0.061 (10)	1.343±0.052 (10)
Brain, Fixed/Body Weight (%)	2.835±0.217 (10)	2.920±0.317 (10)	2.966±0.187 (10)	3.006±0.247 (10)
PND 75 (±5) (Perfused)				
Terminal Body Weight (g)	204.4±12.2 (9)	208.1±16.2 (10)	197.2±11.2 (10)	201.8±12.0 (10)
Brain, Fixed (g)	1.672±0.071 (10)	1.684±0.089 (10)	1.619±0.058 (10)	1.646±0.080 (10)
Brain, Fixed/Body Weight (%)	0.822±0.051 (9)	0.813±0.066 (10)	0.823±0.057 (10)	0.818±0.056 (10)
PND 75 (±5) (Non-Perfused)				
Terminal Body Weight (g)	203.3±11.4 (10)	195.6±17.7 (10)	205.2±18.0 (10)	192.5±13.1 (9)
Brain, Fresh (g)	1.821±0.051 (10)	1.802±0.074 (10)	1.803±0.069 (10)	1.771±0.052 (9)

Parameter	Dietary Level of BYI 02960			
	Control	120 ppm	500 ppm	1200 ppm
Brain, Fresh/Body Weight (%)	0.898±0.042 (10)	0.928±0.084 (10)	0.885±0.090 (10)	0.924±0.061 (9)

* Statistically different from control, $p \leq 0.05$ Values are mean ± S.D. (*n*)

4. Gross brain measurements:

PND 21 Pup Gross Brain Measurements: There were no test substance-related changes in anterior/posterior cerebrum or cerebellum measurements at any dietary level.

PND 75 (± 5 days) Rat Gross Brain Measurements: There were no test substance-related differences from controls in mean anterior/posterior cerebrum or cerebellum lengths of gross brain measurements for PND 75 (± 5 days) rats at any dietary level.

5. Micropathology brain measurements:

The following micropathology measurements were taken on control and 1200 ppm dietary level for PND 21 pups and PND 75 (± 5 days) rats.

SECTION	MEASUREMENT
Forebrain Section	Frontal Cortex
Midbrain Section	Hippocampal Gyrus
Cerebellum/Pons Section	Cerebellum Height

1) PND 21 Pup Micropathology Brain Measurements (mm): There were no test substance-related differences in any of the mean microscopic brain measurement (frontal cortex, hippocampal gyrus, and cerebellum) at highest dietary level of 1200 ppm.

PND 21 Pups	Males		Females	
Brain Anatomical Area	BYI 02960 (ppm)		BYI 02960 (ppm)	
	0	1200	0	1200
Frontal Cortex	1.29	1.26	1.26	1.22
Hippocampal Gyrus	1.18	1.15	1.21	1.15
Cerebellum	3.87	3.90	3.82	3.71

Key: *n* = 8–10/dietary level

2) PND 75 (± 5 days) Rat Micropathology Brain Measurements (mm): There were no test substance-related differences in any of the mean microscopic brain measurement (frontal cortex, hippocampal gyrus, and cerebellum) for PND 75 (± 5 days) rats at highest dietary level of 1200 ppm.

PND 75 (± 5 days) Rats	Males		Females	
Brain Anatomical Area	BYI 02960 (ppm)		BYI 02960 (ppm)	
	0	1200	0	1200
Frontal Cortex	1.28	1.25	1.20	1.18
Hippocampal Gyrus	1.33	1.38	1.30	1.26
Cerebellum	3.91	3.95	3.72	3.76

Key: *n* = 8–10/dietary level

6. Micropathology:

There were no test substance-related brain micropathology changes for PND 21 pups or PND 75 (± 5 days) rats.

III. Conclusions

Dietary exposure to BYI 02960 from GD 6 through LD 21 to mated female Wistar rats resulted in treatment-related effects evident only at the HDT of 1200 ppm (102 mg/kg bw/day) in maternal animals and offspring. Therefore, the NOAEL for both maternal animals and offspring is 500 ppm (42.4 mg/kg bw/day) in this developmental neurotoxicity study.

KIIA 5.8 - Toxicity studies on metabolites

Toxicology study programs for plant and environmental metabolites of BYI 02960 have been performed in accord with EU guidance, with all studies carried out according to current OECD, EU, USEPA and Japanese MAFF testing guidelines. The toxicological properties of two metabolites specific to BYI 02960 and two additional metabolites which are also formed from other agrochemicals are reported in this section.

Difluoroacetic acid (DFA, BCS-AA56716) is a major soil, water, plant and livestock metabolite of BYI 02960. In the rat ADME study, it was found in urine at around 6% of the administered dose and the organ metabolism study showed that DFA was by far the dominating metabolite in the 24 hours samples of plasma, organs and tissues, accounting for more than 50% of the radioactivity. This metabolite is devoid of genotoxic potential; the acute oral LD50 is between 300 and 2000 mg/kg, similar to parent compound. In a 14-day repeat dietary administration range finding study in the rat, the most significant findings were decreased mean glucose concentration in both sexes and an increase in urea concentration was observed in females only. In a 90-day rat study, DFA was administered in the diet to Wistar rats (10/sex/group) at concentrations of 200, 1000 and 6000 ppm. Lower mean glucose concentrations, lower total bilirubin and slightly higher mean urea concentrations were observed in both sexes at all doses. At 6000 and 1000 ppm dose levels, mean body weight, overall body weight gain and food consumption were reduced in both sexes. Lower hemoglobin concentration and lower mean corpuscular volume were observed in females, together with lower mean corpuscular hemoglobin and lower hematocrit, and higher ketone levels were noted in both sexes. A few black foci were also noted in the glandular part of the stomach in both sexes (including one control female), in correlation with a few cases of focal glandular erosion/necrosis observed at the microscopic examination. The minor changes noted in the clinical chemistry determination at the low dose are considered not to be adverse effects of the test substance as they do not represent any functional impairment in the test organism. Therefore, the dose level of 200 ppm (equating to 12.7 and 15.6 mg/kg body weight/day in males and females, respectively) was considered to be a No Observed Adverse Effect Level (NOAEL) in the male and female Wistar rats. When the NOAEL is expressed in BYI 02960 equivalents, it equates to 38 and 47 mg/kg/day in males and females, respectively. Therefore, difluoroacetic acid was not more toxic than BYI 02960 after subchronic administration to the rat. The metabolic changes observed with DFA are also observed with BYI 02960. The decrease in glucose was reversible and appeared to be adaptative as it was no longer significant during the second part of the rat carcinogenicity study. A detailed comparison of the toxicological profile of DFA and BYI 02960 is presented in paragraph KIIA 5.10.

BYI 02960-difluoroethyl-amino-furanone (BCS-CC98193, BYI 02960-DFEAF) is a minor plant metabolite of BYI 02960 and was also observed in the rat ADME study. It accounted for less than 10% of the administered dose in the rat urine. Since the confined rotational crop study with [furanone-4-^{14}C]BYI 02960 indicated rather high residue levels of BYI 02960-difluoroethyl-amino-furanone in leafy crops and the absence of other suitable markers, BCS decided to include this metabolite in the residue definition for the data collection method for target and rotational crops (see KIIA 6.7.1 and KIIA 6.11.1). The subsequent residue studies revealed that BYI 02960-difluoroethyl-amino-furanone is only a minor plant metabolite. However, based on the results of the confined rotational crop study, additional toxicological studies (acute toxicity testing, genotoxicity testing and a subacute rat study) were conducted to show that the toxicological profile of the metabolite is covered by the endpoints

derived from the parent compound. In the *in vitro* genotoxicity package, the Ames and HPRT tests were negative and the chromosome aberration test was positive. Therefore, two *in vivo* studies (*in vivo* micronucleus test and *in vivo* unscheduled DNA synthesis) were conducted, both of which were negative. Based upon the overall findings, BYI 02960-difluoroethyl-amino-furanone can be considered not genotoxic. The acute oral LD_{50} in rats was higher than 2000 mg/kg. The NOAEL of the 28-day rat study was 3000 ppm equating to 243 and 273 mg/kg/day in males and females, respectively based on body weight effects.

Several toxicology studies exist for the two plant metabolites BYI 02960-CHMP (6-chloropyridin-3-ylmethanol) and BYI 02960-6-CNA (6-chloronicotinic acid), which are metabolites common to other pesticides. For BYI 02960-CHMP, the Ames test was negative. The acute oral rat LD_{50} was 1842 mg/kg in males and 1483 mg/kg in females. In a 90-day rat study, BYI 02960-CHMP administered continuously via dietary administration to Sprague Dawley rats (10/sex/group) at concentrations of 160, 800, 4000 and 20 000 ppm resulted in decreases in mean body weights and mean food consumption in both sexes and a statistically significant increases in serum alkaline phosphatase activity in the 20000 ppm group females at study termination only. Histologically, dose-related eosinophilic intranuclear inclusions were seen in the proximal tubular epithelium of kidneys for 20000 ppm males and females and 4000 ppm males. The no observed effects level (NOEL) was 800 ppm (48.9 mg/kg/day) in males, and 4000 ppm (275.9 mg/kg/day) in females. When the NOEL is expressed in BYI 02960 equivalents, it equates to 97.8 and 551.8 mg/kg/day. Therefore, BYI 02960-CHMP was less toxic than BYI 02960 after subchronic administration to the rat.
For BYI 02960-6-CNA (6-chloronicotinic acid) an acute oral rat toxicity study and an Ames test were performed for the registration of acetamiprid. BYI 02960-6-CNA was not genotoxic and not acutely toxic.

For the remaining metabolites, BYI 02960-difuranone and BYI 02960-acetic acid, a set of two *in vitro* genotoxicity tests (the Ames assay and the *in vitro* micronucleus assay) were conducted on each metabolite. The results of these two sets of studies were negative.

The results of all metabolite studies are summarized in the following table.

Table 5.8-01: Summary of toxicity studies with the metabolites

Study	Species	Results
-DFA		
Ames test M-409724-01-2	*Salmonella Typh.*	Negative
In vitro HPRT Locus Gene Mutation Assay M-409727-01-2	Chinese hamster V79 lung cells	Negative
In vitro Chromosome Aberration Test M-409726-01-2	Chinese hamster V79 lung cells	Negative
Rat Acute Oral Study M-393372-01-2	Sprague Dawley Rat	300 mg/kg < LD_{50} < 2000 mg/kg
14-day range-finding study M-414152-01-2	Wistar rat	NOAEL = 500 ppm (equating to 51 mg/kg/day) based on clinical chemistry changes
90-day dietary study M-424611-01-2	Wistar rat	NOAEL = 200 ppm (12.7/15.6 mg/kg bw in M/F) Decreased BW and FC, decreased Hg conc., mean corpuscular volume, mean corpuscular Hg, hematocrit in females, decreased Glc, TBil, increased urea, higher ketones, focal glandular erosion/necrosis
BYI 02960-DFEAF		
Ames test M-409728-01-2	*Salmonella Typh.*	Negative
In vitro HPRT Locus Gene Mutation Assay M-420095-01-2	Chinese hamster V79 lung cells	Negative
In vitro Chromosome Aberration Test M-420108-01-2	Chinese hamster V79 lung cells	Positive in absence of metabolic activation
***In vivo* tests**	**Dose levels**	
Micronucleus Test in male mice - ip administration M-420540-01-2	125, 250 and 500 mg/kg	Negative
Unscheduled DNA synthesis oral administration M-420111-01-2	1000 and 2000 mg/kg	Negative
Rat Acute Oral Study M-409674-01-2	Sprague Dawley Rat	LD_{50} cut off ≥ 2 000 mg/kg/day
Range-finding dietary rat study M-426158-01-2	Wistar Rat	Lower blood glucose concentration from 1280 ppm (equating to 135 mg/kg/day) in females
28-day dietary study M-426136-01-2	Wistar Rat	NOAEL = 3000 ppm (243 and 273 mg/kg/day in males and females, respectively) based on body weight effects.

Table 5.8-01: Summary of toxicity studies with the metabolites (cont'd.)

Study	Species	Results
BYI 02960-CHMP		
Ames test M-195904-01-2	*Salmonella Typh.*	Negative
Rat Acute Oral Study M-195899-01-2	Sprague Dawley Rat	LD_{50} in males = 1842 mg/kg/day LD_{50} in females = 1483 mg/kg/day
90-day dietary study M-195901-01-2	Sprague Dawley Rat	NOEL= 800 ppm (48.9 mg/kg/day) in males and NOEL= 4000 ppm (275.9 mg/kg/day) in females BWG and FC decreases, increase in alkaline phosphatase activity, eosinophilic intranuclear inclusions in proximal tubular epithelium of kidney
BYI 02960-6-CNA		
Ames test M-195932-01-2	*Salmonella Typh.*	Negative
Rat Acute Oral Study M-195930-01-2	Sprague Dawley Rat	LD_{50} > 5000 mg/kg/day in both males and females
BYI 02960-amino-furanone		
Ames test [ADD DART hyperlink]	*Salmonella Typh.*	Negative
In vitro micronucleus assay [ADD DART hyperlink]	Human lymphocytes	Negative
BYI 02960-acetic acid		
Ames test [ADD DART hyperlink]	*Salmonella Typh.*	Negative
In vitro micronucleus assay [ADD DART hyperlink]	Human lymphocytes	Negative

Difluoroacetic acid

In vitro *genotoxicity - Bacterial assay for gene mutation*

Report:	**KIIA 5.8/01, [redacted] A.; 2010**
Title:	Salmonella Typhimurium, Reverse mutation assay with BCS-AA56716
Report No & Document No	1351101 M-409724-01-2
Guidelines:	OECD 471 (1997); EEC Directive N° 440/2008 B13/14 (2008); EPA Health Effects Test Guideline (OPPTS 870.5100; 1998)
GLP	Yes (certified laboratory) except that no analytical analyses were performed during the study

Executive Summary

This study was performed to investigate the potential of BCS-AA56716 (Difluoroacetic acid, metabolite of BYI 02960) (batch N° BCOO 5984-4-11, 99.6% of purity) to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100 and TA 102.
The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations: Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 pg/plate; Experiment II: 33; 100; 333; 1000; 2500; and 5000 pg/plate.
The plates incubated with the test item showed normal background growth in all strains with or without S9 mix in both experiments.
No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in all strains with or without S9 mix in both experiments. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-AA56716 (metabolite of BYI 02960) at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.
Therefore, BCS-AA56716 (DFA, metabolite of BYI 02960) is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

I. Materials and Methods

A. Material

1. Test Material:	BCS-AA56716
Description:	Colorless liquid
Lot/Batch:	BCOO 5984-4-11
Purity:	99.6%
CAS:	381-73-7
Stability of test compound:	No analysis performed during the study
2. Control materials: Negative:	Culture medium
Solvent:	DMSO; stock solution neutralized with 2N NaOH
Positive:	Sodium azide (Serva) for TA 1535 and TA 100 at 10 µg/plate, 4-Nitro-1,2-phenylene diamine (Sigma) for TA 1537 at 50 µg/plate and TA 98 at 10 µg/plate, Methyl methane sulfonate (Merck-Schuchardt) for TA 102 at 3.0 µL/plate, 2-Aminoanthracene (Sigma Aldrich) for the activating effect of the S9 mix in all strains at 2.5 µg/plate for all strains except for TA 102 at 10.0 µg/plate
3. Test organisms:	
Species:	Salmonella typhimurium LT2 mutants
Strain:	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102
Source:	Strains obtained from [redacted], Germany)
4. Test compound concentrations:	
Experiment I:	First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Experiment II:	For TA 1535, TA 1537, TA98, TA100 and TA 102 with or without S9 mix: 33, 100, 333, 1000, 2500 and 5000 µg/tube

B. Study Design and methods

The experimental phase of the study was performed between August 16 to September 03, 2010 at [redacted], Germany.

The Salmonella/microsome test is a screening method which detects point mutations caused by chemical agents *in vitro*. Auxotrophic mutants of Salmonella typhimurium are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

1. Plate incorporation assay (experiment I)

DMSO (0.1 mL) containing BCS-AA56716 or controls were added to glass vessels with 0.1 mL of bacterial cultures grown overnight, 0.5 mL of S9 mix or buffer and 2 mL of soft agar. The mixture was

placed in a waterbath at 45 °C for 30 seconds, shaken and overlaid onto Petri dishes containing solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were scored using the Petri Viewer Mk2. Three plates were used, both with and without S9 mix, for each strain and dose. The doses for the first trial were routinely determined on the basis of a standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses. If less than three doses were used for assessment, at least two repeats were performed.

2. Pre-incubation assay (experiment II)

An independent repeat was performed as pre-incubation of the previously described mixture in a water bath at 37 °C for 60 minutes. At the end of the preincubation period, 2 mL of molten soft agar were added to the tubes, the content mixed and plated onto Petri dishes with solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were also scored using an automated colony counter.

3. Assessment criteria

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100 and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed. A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration. An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and discussion

The plates incubated with the test item showed normal background growth in all strains in both independent experiments with and without S9 mix.
No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.
No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-AA56716 (DFA metabolite of BYI 02960) at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix).
There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.
Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

III. Conclusions

No indication of mutagenic effects of BCS-AA56716 (DFA, metabolite of BYI 02960) could be found at assessable doses of up to 5000 µg/plate in any of the Salmonella typhimurium strains used in the assay.

In vitro *genotoxicity - Test for clastogenicity in mammalian cells*

Report:	**KIIA 5.8/02, [redacted] C.; 2010**
Title:	BCS-AA56716 (Metabolite of BYI 02960), *In vitro* chromosome aberration test with Chinese Hamster V79 cells
Report No & Document No	1351103 M-409726-01-2
Guidelines:	OECD 473 (1997); EEC Directive 440/2008 Method B10; EPA Health Effects Test Guideline (OPPTS 870.5375; 1998)
GLP	Yes (certified laboratory)

Executive Summary

In this *in vitro* assessment of the clastogenic potential of BCS-AA56716 (batch BCOO 5984-4-11, 99.6% of purity), Chinese Hamster V79 cells were exposed to BCS-AA56716 at 3.8, 7.5, 15, 30, 60, 120, 240, 480 and 960 µg/mL (960 µg/mL = 10 mM), diluted in dimethyl sulphoxide (DMSO). For each dose level, duplicate cultures were used in both the presence and absence of a metabolic activation system (S9 mix). DMSO was also used as a negative control. Ethylmethane sulfonate, which produces crosslinks in the DNA, and cyclophosphamide, which induces chromosomal damage after metabolic activation, were used as positive controls. After 4 hours treatment, the medium was changed and the cells were harvested 14 hours later. An additional experiment was performed using continuous treatment for 18 hours, harvest at the same time, at BCS-AA56176-concentrations of 60, 120, 240, 480 and 960 µg/mL. Colcemid was added to each flask two hours prior to harvest to arrest the cells in a metaphase-like stage of mitosis.

In the absence and presence of S9 mix neither test item precipitation nor relevant cytotoxicity were observed up to the highest required concentration.
In both experiments no clastogenicity was observed at the concentrations evaluated either with or without metabolic activation. In Experiment I in the absence of S9 mix an isolated increase in the number of aberrant cells, excluding gaps (4.5%, slightly exceeding the total laboratory's historical solvent control data range (0.0 - 4.0% aberrant cells, excluding gaps) was observed after treatment with 480.0 µg/mL. This observation is regarded as biologically irrelevant, since the value was not statistically significantly increased and no increase was observed at the highest applied dose of 960 µg/mL.
No relevant evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the controls.
Appropriate mutagens were used as positive controls. They induced statistically significant increases ($p < 0.05$) in cells with structural chromosome aberrations.

In conclusion, BCS-AA53716 (DFA, metabolite of BYI 02960) is considered to be non-clastogenic in this chromosome aberration test in the absence and presence of metabolic activation, when tested up to the highest required test item concentration.

I. Materials and Methods

A. Material

1. Test Material:	BCS-AA56716
Description:	Colorless liquid
Lot/Batch:	Batch BCOO 5984-4-11
Purity:	99.6%
CAS:	381-73-7
Stability of test compound:	No analysis performed during the study

2. Control materials:
Negative: Culture medium
Solvent: DMSO for BCS-AA56716
Positive: Ethylmethane sulfonate (Acros organics, Belgium) without S9 mix at 1000 µg/mL for experiment I, and 600 µg/mL for experiment II
Cyclophosphamide (Sigma-Aldrich, Germany) with S9 mix at 1.4 µg/mL

3. Test organisms:
Cell line: Chinese hamster V79 lung cells
Source: Cells supplied by [redacted] Germany
Culture condition: Incubation performed at 37 °C in a humidified atmosphere with about 1.5% CO_2

4. Test compound concentrations:
In experiment I: Exposure period 4 hours with or without S9 mix, BCS-AA56716 was used at 3.8, 7.5, 15, 30, 60, 120, 240, 480 and 960 µg/mL
In experiment II: 18 hours exposure without S9 mix, BCS-AA56716 was used at 60, 120, 240, 480 and 960 µg/mL
In experiment II: 4 hours exposure with S9 mix, BCS-AA56716 was used at 60, 120, 240, 480 and 960 µg/mL

B. Study design and methods

The experimental phase of the study was performed from August 04 to October 01, 2010 at [redacted] Germany.

The *in vitro* cytogenetic test is a mutagenicity test system for the detection of chromosome aberrations in cultured mammalian cells. The test is designed to detect structural aberrations (chromatid and chromosome aberrations) in cells at their first post-treatment mitosis.

1. Determination of cytotoxicity

960.0 µg/mL of BCS-AA56716 (approx. 10 mM) was selected as the top concentration for treatment of the cultures in the pre-test. Test item concentrations between 3.8 and 960.0 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. No precipitation of the test item was observed.

Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.
Dose selection of Experiment II was influenced by the results obtained in Experiment I. No cytotoxicity was observed up to the highest applied concentration. Therefore, 960.0 µg/mL was chosen as top treatment concentration for Experiment ll.

2. Seeding of the cultures
Thawed stock-cultures were propagated at 37 °C in 80 cm^2 plastic flasks. About 5 x 10^6 cells per flask were seeded in 15 mL of MEM (minimal essential medium) containing Hank's salts and 10 % (v/v) fetal bovine serum (FBS). Additionally, the medium was supplemented with neomycin (5 µg/mL), Hepes (25 mM) and amphotericin B (2.5 µg/mL). The cells were subcultured twice a week. The cell cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % carbon dioxide (98.5 % air).

Exponentially growing stock cultures more than 50% confluent were rinsed with Ca-Mg-free salt solution containing 8000 mg/L NaCl, 200 mg/L KCl, 200 mg/L KH_2PO4 and 50 mg/L Na_2HPO_4. Afterwards the cells were treated with trypsin-EDTA solution at 37 °C for approx. 5 minutes. Then, by adding complete culture medium including 10 % (v/v) FBS the enzymatic treatment was stopped and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.5 % (w/v) in Ca-Mg-free salt solution. The cells were seeded into Quadriperm dishes, which contained microscopic slides, into each chamber 1 x 10^4 - 6 x 10^4 cells were seeded with regard to the preparation time.

3. Treatment protocol
Exposure period 4 hours
The culture medium of exponentially growing cell cultures was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium were added. Concurrent solvent and positive controls were performed. After 4 hours the cultures were washed twice with saline. The cells were then cultured in complete medium containing 10 % (v/v) FBS for the remaining culture time of 14 hours.

Exposure period 18 hours
The culture medium of exponentially growing cell cultures was replaced with complete medium containing 10 % (v/v) FBS including the test item without S9 mix. The medium was not changed until preparation of the cells. Concurrent solvent and positive controls were performed.

4. Preparation of the cultures
Colcemid was added to the culture medium (0.2 µg/mL) 15.5 hours after the start of the treatment, The cells were treated, 2.5 hours later, on the slides in the chambers with hypotonic solution (0.4% KCl) for 20 min at 37 °C. After incubation in the hypotonic solution the cells were fixed with a mixture of methanol and glacial acetic acid (3 : 1 parts, respectively). After preparation the cells were stained with Giemsa and labelled with a computer-generated random code to prevent scorer bias.

5. Evaluation of cell numbers

The evaluation of cytotoxicity indicated by reduced cell numbers was made after the preparation of the cultures on spread slides. The cell numbers were determined microscopically by counting 10 defined fields per coded slide. The cell number of the treatment groups is given in percentage compared to the respective solvent control.

6. Analysis of metaphase cells

Evaluation of the cultures was performed using NIKON microscopes with 100x objectives. Breaks, fragments, deletions, exchanges, and chromosome disintegrations were recorded as structural chromosome aberrations. Gaps were recorded as well but not included in the calculation of the aberration rates. At least 100 well spread metaphases per culture were evaluated for cytogenetic damage on coded slides, except for the positive control in Experiment I without metabolic activation, where only 50 metaphases were evaluated. Only metaphases with characteristic chromosome numbers of 22 ± 1 were included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined. In addition, the number of polyploid cells in 500 metaphases per culture was determined (% polyploid metaphases: in the case of this aneuploid cell line polyploid means a near tetraploid karyotype).

7. Evaluation criteria

A test item was classified as non-clastogenic if:

- The number of induced structural chromosome aberrations in all evaluated dose groups was in the range of the laboratory's historical control data,

and/or

- no significant increase of the number of structural chromosome aberrations was observed.

A test item is classified as dastogenic if:

- The number of induced structural chromosome aberrations is not in the range of the laboratory's historical control data,

and

- either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

Statistical significance was confirmed by means of the Fisher's exact test ($p < 0.05$). However, both biological and statistical significance should be considered together. If the criteria mentioned above for the test item were not clearly met, the classification with regard to the historical data and the biological relevance was discussed and/or a confirmatory experiment was performed.

Although the inclusion of the structural chromosome aberrations was the purpose of this study, it was important to include the polyploids and endoreduplications.

8. Assessment criteria

The chromosome aberration test was considered acceptable if it met the following criteria:

- The number of structural aberrations found in the solvent controls fell within the range of the laboratory's historical control data.

- The positive control substances produced significant increases in the number of cells with structural chromosome aberrations, which were within the range of the laboratory's historical control data.

II. Results and discussion

The test item BCS-AA56716 (DFA metabolite of BYI 02960), dissolved in DMSO was assessed for its potential to induce structural chromosome aberrations in *V79* cells of the Chinese hamster *in vitro* in the absence and presence of metabolic activation by S9 mix.
Two independent experiments were performed. In Experiment I the exposure period was 4 hours with and without metabolic activation. In Experiment II the exposure period was 4 hours with S9 mix and 18 hours without S9 mix. The chromosomes were prepared 18 hours after start of treatment with the test item. In each experimental group two parallel cultures were set up. At least 100 metaphases per culture were evaluated for structural chromosome aberrations, except for the positive control in Experiment I without S9 mix, where only 50 metaphases were evaluated.

Neither precipitation of the test item in the culture medium nor relevant increase in the osmolarity was observed (Exp. I: solvent control 393 mOsm versus 399 mOsm at 960.0 µg/mL; Exp. II: solvent control 394 mOsm versus 399 mOsm at 950.0 µg/mL). The pH value of the culture medium containing the test item at concentrations of 480.0 and 980.0 µg/mL was adjusted to 7.0 - 7.5 by addition of 2N NaOH.
No relevant cytotoxic effects indicated by reduced mitotic indices or reduced cell numbers to approximately 50% were observed after treatment with the test item. However, the maximum applied dose of 960.0 µg/mL in Experiment II in the presence of S9 mix lead to a reduction of cell numbers to 64.9% of the solvent control.
In both experiments, in the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying structural chromosome aberrations was observed. In Experiment I in the absence of S9 mix an isolated single increase in the number of aberrant cells, excluding gaps (4.5%) slightly exceeding the total laboratory's historical solvent control data range (0.0 - 4.0% aberrant cells, excluding gaps) was observed after treatment with 480.0 µg/mL. This observation was regarded as biologically irrelevant, since the value was not statistically significantly increased and no increase was observed at the highest applied dose of 960 µg/mL.
No relevant evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the controls.
Either EMS (1000.0 and 600.0 µg/mL) or CPA (1.4 µg/mL) were used as positive controls and showed distinct increases in the number of cells with structural chromosome aberrations.

Table 5.8-02: Summary of the results of the chromosomal aberration study with BCS-AA56716

Experiment	Test item concentration	Cell number in % of control	Mitotic indices in % of control	Aberrant cells in %		
				Including gaps £	Excluding gaps £	With exchanges
Exposure period of 4 hours without S9 mix						
I	DMSO 0.5%	100.0	100.0	3.0	3.0	1.5
	EMS 1000 µg/mL $	n.t.	94.0	39.0	37.0 *	22.0
	240 µ	111.8	91.7	3.8	3.5	0.8
	480 µ	113.7	106.0	4.8	4.5	1.5
	960	115.5	90.5	3.5	2.0	0.0
Exposure period of 18 hours without S9 mix						
II	DMSO 0.5%	100.0	100.0	2.5	2.0	0.0
	EMS 600 µg /mL	n.t.	89.9	19.5	17.0 *	6.0
	240	87.3	107.5	1.0	0.5	0.0
	480	84.0	106.3	0.0	0.0	0.0
	960	107.1	108.2	1.5	1.5	0.0
Exposure period of 4 hours with S9 mix						
I	DMSO 0.5%	100.0	100.0	3.5	3.5	0.0
	CPA 1.4 µg /mL	n.t.	71.4	9.0	8.5 *	4.5
	240	121.3	106.9	1.5	1.0	0.0
	480	99.7	112.8	1.0	1.0	0.0
	960	119.8	103.9	3.5	3.5	0.0
II	DMSO 0.5%	100.0	100.0	2.5	2.0	0.5
	CPA 1.4 µg /mL	n.t.	98.2	15.0	12.0 *	4.0
	240	93.0	102.7	2.0	2.0	0.5
	480	85.5	100.5	2.0	2.0	1.0
	960	64.9	87.7	1.5	1.0	0.0

£ : inclusive cells carrying exchanges $: Evaluation of 50 metaphases per culture
µ : evaluation of 200 metaphases per culture n.t. : not tested * : $p < 0.05$

III. Conclusions

In conclusion, it can be stated that under the experimental conditions reported, the test item BCS-AA56716 (DFA metabolite of BYI 02960) did not induce structural chromosome aberrations in *V79* cells (Chinese hamster cell line) when tested up to the highest test item concentration required by the guideline.

In vitro *genotoxicity - Test for clastogenicity in mammalian cells*

Report:	**KIIA 5.8/03, [redacted]C.; 2010**
Title:	BCS-AA56716 (metabolite of BYI 02960), Gene mutation assay in Chinese Hamster V79 cells *in vitro* (V79/HPRT)
Report No & Document No	1351102 M-409727-01-2
Guidelines:	OECD 476 (1997); EEC Directive 440/2008 Method B17 (2008); EPA Health Effects Test Guideline (OPPTS 870.5300; 1998)
GLP	**Yes (certified laboratory)**

Executive Summary

The study was performed to investigate the potential of BCS-AA56716 (DFA, metabolite of BYI 02960) (Batch N° BCOO 5984-4-11, 99.6% of purity) to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster.

In the range finding pre-experiment test item concentrations between 7.3 and 9400 pg/mL (~ 9.8 mM) were used to evaluate toxicity in the presence (4 hours treatment) and absence (4 and 24 hours treatment) of metabolic activation. No relevant toxic effect occurred up to the maximum concentration with and without metabolic activation following 4 and 24 hours treatment. Neither precipitation nor phase separation was observed up to the maximum concentration of 9600 pg/mL. There was no relevant shift of pH and osmolarity of the medium even in the stock solution of the test item. Therefore, the maximum concentration in the main experiments was 9600 pg/mL corresponding to 10 mM of the test item.

The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours at concentrations ranging from 60 to 960 mg/mL. The second experiment was performed with a treatment time of 4 hours with metabolic activation at concentrations ranging from 120 to 960 mg/mL and 24 hours without metabolic activation at concentrations ranging from 30 to 960 mg/mL.

No substantial and reproducible dose dependent increase of the mutation frequency was observed in both main experiments.

Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies and thus, showed the sensitivity of the test item and the activity of the metabolic activation system.

In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells. Therefore, BCS-AA56716 (metabolite of BYI 02960) is considered to be non-mutagenic in this HPRT assay.

I. Materials and Methods

A. Material

1. Test Material:	BCS-AA56716
Description:	Colorless liquid
Lot/Batch:	BCOO 5984-4-11

Purity:		99.6%
CAS:		381-73-7
Stability of test compound:		No analysis performed during the study
2. Control materials:	Negative:	Culture medium: Eagle's minimal essential medium supplemented with Hank's salts, 5 mg/mL of Neomycin, 1% of Amphotericin B and 10% foetal calf serum (FCS)
	Solvent:	DMSO for BCS-AA56716 and Dimethylbenzanthracene not exceeding 0.5% (v/v) in the culture medium. No solvent needed for ethyl methanesulfonate as it is a liquid.
	Positive:	Ethyl methanesulfonate (EMS), a directly alkylating agent, used at a final concentration of 150 µg/mL (1.2 mM) in non-activation trials. Dimethylbenzanthracene (DMBA), promutagen requiring a metabolic activation, used at a final concentration of 1.1 µg/mL for trials with S9 mix.
3. Test organisms:		
Cell line:		Chinese hamster V79 lung cells
Source:		Cells supplied by [redacted], [redacted] Germany
Culture condition:		Incubation performed at 37 °C in a humidified atmosphere with about 1.5% CO_2.
4. Test compound concentrations:		BCS-AA56716 was used from 7.3 to 940.0 µg/mL in the range-finding pre-experiment and at 30, 60, 120, 240, 480 and 960 µg/mL in the mutagenic assays
5. Metabolic activation:		The S9 fraction was isolated from the livers of Phenobarbital/β-naphthoflavone induced male Wistar rats. The protein concentration of the S9 preparation was 35 mg/mL in the pre-experiment and 33.7 mg/mL in experiments I and II.

B. Study Design and methods

The experimental phase of the study was performed From July 27 to September 10, 2010 at [redacted], Germany.

The selection of V79 forward mutations is based on the resistance of induced mutants to the purine analogue 6-thioguanine (6-TG). This resistance is a result of a mutation at the X-chromosome-linked HPRT locus rendering the cells unable to use 6-TG for DNA synthesis. Therefore, cell colonies formed in the presence of 6-TG are considered to represent mutants at the HPRT gene.

1. Determination of cytotoxicity

The general culture conditions and experimental conditions in this pre-test were the same as described for the mutagenicity experiment below. In this pre-test the colony forming ability of approximately 500 single cells (duplicate cultures per concentration level) after treatment with the test item was observed and compared to the controls.
Toxicity of the test item is indicated by a reduction of the cloning efficiency (CE).

2. Treatment protocol

Thawed stock cultures were propagated at 37 °C in 80 cm^2 plastic flasks. About 5 x 10^5 cells were seeded into each flask with 15 mL of MEM (minimal essential medium) containing Hank's salts, neomycin (5 pg/mL) and Amphotericin B (1 %). The cells were sub-cultured twice weekly. The cell cultures were incubated at 37 °C in a 1.5% carbon dioxide atmosphere (98.5% air). For the selection of mutant cells the complete medium was supplemented with 11 pg/mL 6-thioguanine.
Two days (experiment I) or three days (experiment II) after sub-cultivation stock cultures were trypsinized at 37 °C for 5 minutes. Then the enzymatic digestion was stopped by adding complete culture medium and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.2% in Ca-Mg-free salt solution.
The cell suspension was seeded into plastic culture flasks. Approximately 1.5 x 10^6 (single culture) and 5 x 10^2 cells (in duplicate) were seeded in MEM with 10% FBS (complete medium) for the determination of mutation rate and toxicity, respectively.
After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 mL/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps. In the second experiment the cells were exposed to the test item for 24 hours in complete medium, supplemented with 10% FBS, in the absence of metabolic activation.

Three days (experiment II) or four days (experiment I) after treatment 1.5 x 10^6 cells per experimental point were sub-cultivated in 175 cm^2 flasks containing 30 mL medium. Following the expression time of 7 days five 80 cm^2 cell culture flasks were seeded with about 3 - 5 x 10^5 cells each in medium containing 6-TG. Two additional 25 cm^2 flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2 for about 8 days. The colonies were stained with 10% methylene blue in 0.01% KOH solution. The stained colonies with more than 50 cells were counted. In doubt the colony size was checked with a preparation microscope (Nikon, 40407 Düsseldorf, Germany).

3. Acceptance criteria

The gene mutation assay was considered acceptable if it meets the following criteria:

- The numbers of mutant colonies per 10^6 cells found in the solvent controls fell within the laboratory historical control data range
- The positive control substances must produce a significant increase in mutant colony frequencies
- The cloning efficiency II (absolute value) of the solvent controls must exceed 50%.

The data of this study comply with the above mentioned.

4. Assessment criteria

A test item was classified as positive if it induced either a concentration-related increase of the mutant frequency or a reproducible and positive response at one of the test points.
A test item producing neither a concentration-related increase of the mutant frequency nor a reproducible positive response at any of the test points was considered to be non-mutagenic in this system.
A positive response was described as follows:
A test item was classified as mutagenic if it reproducibly induced a mutation frequency that was three times above the spontaneous mutation frequency at least at one of the concentrations in the experiment.
The test item was classified as mutagenic if there was a reproducible concentration-related increase of the mutation frequency. Such evaluation may be considered also in the case that a threefold increase of the mutant frequency was not observed.
However, in a case by case evaluation this decision depends on the level of the corresponding solvent control data. If there was by chance a low spontaneous mutation rate within the laboratory's historical control data range, a concentration-related increase of the mutations within this range has to be discussed. The variability of the mutation rates of solvent controls within all experiments of this study was also taken into consideration.

5. Statistical analysis

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. The number of mutant colonies obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

II. Results and discussion

No relevant toxic effect occurred up to the maximum concentration with and without metabolic activation following 4 and 24 hours treatment. The test medium was checked for precipitation or phase separation at the end of each treatment period (4 or 24 hours) prior to removal to the test item. Neither precipitation nor phase separation was observed up to the maximum concentration of 960.0 µg/mL.
There was no relevant shift of pH and osmolarity of the medium even in the stock solution of the test item. Therefore, the maximum concentration in the main experiments was 960.0 µg/mL corresponding to 10 mM of the test item.

No relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration. The induction factor exceeded the threshold of three times the corresponding solvent control in the first culture of the first experiment without metabolic activation in all dose groups. This effect however, was judged to be due to the rather low solvent controls of 5.8 mutant colonies/10^6 cells. The induction factor was well below 3.0 in the respective parallel culture. Additionally, several mutant frequencies (33.9 - 54.4 mutant colonies/10^6 cells) exceeded the respective historical range of solvent controls. This effect is considered as not being biologically relevant as the mutation frequencies did not exceed the historical data range in the respective parallel culture or the induction factor did not exceed the threshold of three times the solvent control on a reproducible basis.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequency. No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in any of the experimental groups.

In both experiments of this study (with and without metabolic activation) the range of the solvent controls was from 5.8 up to 45.3 mutant colonies per 10^6 cells; the range of the groups treated with the test item was from 8.6 up to 54.4 mutant colonies per 106 cells. The highest solvent control after treatment with S9 mix in experiment I and without S9 mix in experiment II (45.3 and 38.4 mutant colonies per 106 cells, respectively) exceeded the historical range of solvent control slightly (3.0 - 33.2 and 3.9 - 31.5 colonies per 10^6 cells). However, this effect was judged as irrelevant since it is very minor and the corresponding solvent control remained well within the range of historical controls. EMS (150 µg/mL) and DMBA (1.1 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies.

Table 5.8-03: Mean mutant colonies per 10^6 cells in experiment I

Test groups	Concentration µg/mL	S9 mix	Culture I		Culture II	
			Mutant colonies per 10^6 cells	Induction factor	Mutant colonies per 10^6 cells	Induction factor
Control DMSO	-	-	5.8	1.0	24.8	1.0
Positive control EMS	150.0	-	268.6	46.4	168.9	6.8
BCS-AA56716	60.0	-	34.5	6.0	22.8	0.9
	120.0	-	38.5	6.7	35.5	1.4
	240.0	-	27.2	4.7	17.1	0.7
	480.0	-	38.3	6.6	16.4	0.7
	960.0	-	24.0	4.1	22.1	0.9
Control DMSO	-	+	45.7	1.0	25.6	1.0
Positive control DMBA	150.0	+	1232.2	27.2	1000.4	39.0
BCS-AA56716	60.0	+	18.4	0.4	54.4	2.1
	120.0	+	8.6	0.2	21.2	0.8
	240.0	+	20.9	0.5	37.9	1.5
	480.0	+	34.5	0.8	10.1	0.4
	960.0	+	41.1	0.9	35.6	1.4

Table 5.8-04: Mean mutant colonies per 10^6 cells in experiment II

Test groups	Concentration µg/mL	S9 mix	Culture I		Culture II	
			Mutant colonies per 10^6 cells	Induction factor	Mutant colonies per 10^6 cells	Induction factor
Control DMSO	-	-	38.4	1.0	14.5	1.0
Positive control EMS	150.0	-	534.4	13.9	410.3	28.3
BCS-AA56716	30.0	-	28.8	0.8	13.7	0.9
	60.0	-	22.7	0.6	23.4	1.6
	120.0	-	22.3	0.6	11.8	0.8
	240.0	-	25.3	0.7	28.2	1.9
	960.0	-	22.5	0.6	22.1	1.5
Control DMSO	-	+	24.5	1.0	27.0	1.0
Positive control EMS	150.0	+	1337.5	54.7	1185.9	43.9
BCS-AA56716	120.0	+	23.1	0.9	24.1	0.9
	240.0	+	26.5	1.1	37.1	1.4
	480.0	+	52.8	2.2	27.0	1.0
	960.0	+	33.9	1.4	27.0	1.0

III. Conclusions

In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.
Therefore, BCS-AA56716 (metabolite of BYI 02960) is considered to be non-mutagenic in this HPRT assay.

Acute oral toxicity

Report:	**KIIA 5.8/04, [redacted] C.; 2010**
Title:	BCS-AA56716, Acute toxicity in the rats, "Acute toxic class method."
Report No & Document No	37066 TAR M-393372-01-2
Guidelines:	OECD 423 (2001); EEC Directive 440/2008, Method B.1.tris (2008)
GLP	Yes (certified laboratory)

Executive Summary

In an acute oral toxicity study using a stepwise procedure, three groups of three fasted, young adult female Sprague Dawley rats were given successively a single oral dose of BCS-AA56716 (batch BCOO 5984-4-11, 99.6 % purity) in purified water of 300, 2 000 and then 300 mg/kg bw and were observed for 14 days.
At the 300 mg/kg dose-level no deaths occurred (three females then confirmation on three other females). Loud breathing was recorded in 1/6 females on day 3 only. No other clinical signs were recorded. A lower body weight gain (28 g *vs.* 41 ± 9 g in control data base) was noted in 1/6 females between day 1 and day 8. The body weight gain of this female returned to normal thereafter. At necropsy, no apparent abnormalities were observed in any animals.

At the 2000 mg/kg dose-level (three females), two females were found dead 1 hour after treatment. Hypoactivity, dyspnea and/or lateral recumbency were observed within 25 minutes prior to their deaths. In the surviving animal, sedation then hypoactivity, lateral recumbency, dyspnea, piloerection and staggering gait were noted from day 1 until day 9, associated with a body weight loss of 8% between day 1 and day 8. The body weight gain of this female return to normal thereafter. At necropsy, no apparent abnormalities were observed in this female.

Under the experimental conditions of this study, the oral LD_{50} of the test item, BCS-AA56716, was found to be between 300 and 2000 mg/kg in rats. According to the classification criteria laid down in Council Directive 67/548/EEC (and subsequent adaptations), concerning the potential toxicity by oral route, the test item should be assigned the symbol Xn, the indication of danger "Harmful" and the risk phrase R 22: "Harmful if swallowed".

I. Materials and Methods

A. Material

1. Test Material:	BCS-AA56716
Description:	Colorless liquid
Lot/Batch:	BCOO 5984-4-11
Purity:	99.6%
CAS:	381-73-7
Stability of test compound:	No analysis performed during the study
2. Vehicle and /or positive control:	Purified water
3. Test animals:	
Species:	Rat
Strain:	Rj: SD (IOPS Han).
Age:	8 weeks approximately
Weight at dosing:	208 ± 13 g
Source:	[redacted] France
Acclimation period:	At least 5 days
Diet:	[redacted] R/M-H pelleted diet, batch No. 3713952 ([redacted]), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were group caged conventionally in polycarbonate cages on autoclaved sawdust
Environmental conditions:	Temperature: 22 ± 2 °C
	Humidity: 50 ± 20%
	Air changes: Approximately 12 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates

July 16 to August 18, 2010 performed at [redacted] France).

2. Animal assignment and treatment

The substance was tested using a stepwise procedure, each step using three female rats. The animals were assigned to their groups by randomization based on evenly distributed chance numbers. Following an overnight fast (18 hours), the first group received a single dose of 300 mg/kg of BCS-AA56716 (99.6% purity) by gavage. The test substance was administered in purified water at a volume of 10 mL/kg bw. After the first assay, as no deaths occurred, another assay was carried out on three animals at the next higher dose-level (2000 mg/kg). After the second assay, as 2/3 animals died, the results were confirmed in three other females at the dose-level of 300 mg/kg. Clinical signs and mortality rates were determined several times on the day of administration and subsequently at least once daily for an observation period of at least 14 days. Body weights were recorded on days 1, 8 and 15. On day 15,

surviving animals were sacrificed and all animals were necropsied and examined for gross pathological changes.

3. Statistics

The data did not warrant statistical analysis.

II. Results and discussion

A. Mortality

At the 300 mg/kg dose-level (three females then confirmation on three other females), no deaths occurred. At the 2000 mg/kg dose-level (three females), two females were found dead 1 hour after treatment. Hypoactivity, dyspnea and/or lateral recumbency were observed within 25 minutes prior to their deaths.

B. Clinical observations

In the surviving animal at 2000 mg/kg, sedation then hypoactivity, dyspnea, lateral recumbency, piloerection and staggering gait were noted from day 1 until day 9. At 300 mg/kg, loud breathing was recorded in 1/6 females on day 3. No other clinical signs were noted.

C. Body weight

When compared to CIT historical control data, a lower body weight gain (28 g *vs.* 41 ± 9 g in control data base) was noted in 1/6 females given 300 mg/kg between day 1 and day 8. The body weight gain of this female returned to normal thereafter. The body weight gain of the other animals was not affected by the test item-treatment.
A body weight loss of 8% (185 g *vs.* 201 g) was noted in the surviving female given 2000 mg/kg, between day 1 and day 8. The body weight gain of this female return to normal thereafter.

D. Necropsy

No abnormalities were observed at gross necropsy.

III. Conclusions

Under the experimental conditions of this study, the oral LD_{50} of the test item, BCS-AA56716, was found to be between 300 and 2000 mg/kg in rats.

Oral 14-day toxicity in the rat

Report:	**KIIA 5.8/05; [redacted] P.; 2011**
Title:	BCS-AA56716 (Difluoroacetic acid), Preliminary 14-day toxicity study in the rat by dietary administration
Report No & Document No	SA 10323; M-414152-01-2
Guidelines:	Not applicable (only preliminary and explorative study design)
GLP	Study not performed under GLP, but laboratory GLP-certified

Executive Summary

BCS-AA56716 (difluoroacetic acid), a metabolite of BYI 02960 (batch number BC00 5984-4-11; a colorless liquid, 96.7% w/w purity) was administered continuously via the diet to groups of Wistar rats (5/sex/group) for at least 14 days at concentrations of 500, 2000 and 8000 ppm (equating to approximately 48, 187 and 745 mg/kg body weight/day in males and 51, 201 and 800 mg/kg body weight/day in females). A similarly constituted group received untreated diet and served as a control. Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight and food consumption were recorded once weekly. Hematology and clinical chemistry parameters were determined at the end of the study. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically.

BCS-AA56716 dietary administration at dose levels of 8000, 2000 and 500 ppm to males and females Wistar rats for at least 14 days induced no treatment-related changes with regard to survival, clinical signs, food consumption, hematological parameters, macroscopic observation and microscopic examination.

At 8000 ppm, mean body weight was similar to the controls in both sexes on study Day 8 and was slightly reduced by 4% in males and 3% in females on study Day 15 (not statistically significant) in comparison to the controls. This slight effect was attributable to a mean cumulative body weight gain reduced by 13% in males and 12% in females throughout the study (not statistically significant) when compared to the controls. Clinical chemistry revealed a mean glucose concentration reduced by 54% in males and 45% in females ($p < 0.01$) compared to the controls. In addition, mean urea concentration was 27% higher in females (not statistically significant) in comparison to the controls. At necropsy, mean terminal body weight was lower by 6% in males and 7% in females (not statistically significant), compared to the controls.

At 2000 ppm, mean body weight parameters were unaffected by treatment in males, whilst in females a marginal reduction in mean cumulative body weight gain was noted throughout the study (- 12% compared to the controls, not statistically significant). Clinical chemistry revealed a mean glucose concentration reduced by 41% in males and 48% in females ($p < 0.01$) compared to the controls. In addition, mean urea concentration was 23% higher in females (not statistically significant) in comparison to the controls. At necropsy, mean terminal body weight was lower by 6% in females only (not statistically significant) compared to the controls. The change observed in males at this dose level was considered not to be adverse in view of its isolated occurrence and absence of associated histological findings.

At 500 ppm, the only changes noted for clinical chemistry consisted of a mean glucose concentration reduced by 34% in males and 42% in females ($p < 0.01$), together with mean urea concentration higher

by 23% in females (not statistically significant) in comparison to the controls. These few changes were considered not to be adverse in view of their isolated occurrence and absence of associated histological findings.

In conclusion, the dietary level of 2000 ppm (corresponding to 187 mg/kg body weight/day) was considered to be a NOAEL in males, and the dietary level of 500 ppm (corresponding to 51 mg/kg body weight/day) was considered to be a NOAEL in females.

I. Materials and Methods

A. Material

1. Test Material:	BCS-AA56716
Description:	Colorless liquid
Lot/Batch:	BCOO 5984-4-11
Purity:	96.7%
CAS:	381-73-7
Stability of test compound:	No analysis were performed on the dietary formulations
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age:	6 weeks approximately
Weight at dosing:	189 to 208 g for the males; 148 to 166 g for the females
Source:	[redacted] France
Acclimation period:	6 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 55 ± 15% Air changes: Approximately 10 to15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates

29 September 2010 to 20 October 2010 at [redacted], France.

2. Animal assignment and treatment

There were 5 rats per sex per dose group. Animals were assigned to dose groups using a randomization by weight. BCS-AA56716 was administered in the diet for 14 days to Wistar rats at the following doses: 0, 500, 2000 and 8000 ppm (equating to approximately 48, 187 and 745 mg/kg/day in males and 51, 201 and 800 mg/kg/day in females). A negative control group received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

3. Diet preparation and analysis

BCS-AA56716 was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. When not in use, the diet formulations were stored at approximately -18 °C. No analyses were performed on the dietary formulations.

Table 5.8-05: Study design

Test Group	Diet Concentration (ppm)	Animals assigned	
		Male	Female
1	0	5	5
2	500	5	5
3	2000	5	5
4	8000	5	5

4. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using PathTox System V4.2.2. (Module Enhanced Statistics).

B. Methods

1. Observations
All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). All animals were observed for clinical signs at least once daily. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight
Each animal was weighed at least weekly during the acclimatization period, on the first day of test substance administration, then at weekly intervals throughout the treatment period. Additionally, diet fasted animals were weighed before scheduled necropsy (Terminal body weight).

3. Food consumption and compound intake
Food consumption was recorded weekly, the weekly mean achieved dosage intake for each week and for weeks 1 to 2 was calculated.

4. Clinical pathology
Blood sampling
On Study Day 16, blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane (Baxter, Maurepas, France). Blood was collected on EDTA for hematology, on clot activator (for serum) for clinical chemistry and on sodium citrate for coagulation parameters.

Hematology
Red blood cell count, haemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count were assayed using an Advia 120 (Siemens, Eragny, France).
A blood smear was prepared and stained using May-Grünwald-Giemsa method. It was examined when the results of Advia 120 determinations were abnormal.
Prothrombin time (10) was assayed on an ACL ElitePro (Instrumentation Laboratory, Paris, France).

Clinical chemistry
Any significant change in the general appearance of the serum was recorded.

Total bilirubin, glucose, urea, creatinine, total protein, albumin, total cholesterol and triglycerides concentrations, and aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed using an Advia 1650 (Siemens, Eragny, France).

5. Sacrifice and pathology
On Study Day 16, all animals from all groups were sacrificed by exsanguination whilst under deep anesthesia (Isoflurane inhalation). Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities.

Macroscopic abnormalities were recorded, sampled and examined microscopically. The following organs were weighed: Adrenal glands, brain, kidney, liver, ovary, spleen, testis, thyroid gland (with parathyroid gland), uterus (including cervix) and pituitary gland. Paired organs were weighed together. The same organs plus epididymis, vagina and macroscopic findings were sampled and fixed by immersion in neutral buffered 10% formalin with the exception of testis and epididymis that was fixed in Davidson's fixative. Histological sections were prepared for all animals in all groups and stained with hematoxylin and eosin. Histopathological examinations were performed on all tissues except for the parathyroid gland, for all animals in the control and high dose group. Kidney, liver, thyroid gland and significant macroscopic findings of all animals were examined in the intermediate dose groups.

II. Results and discussion

A. Observations

1. Clinical signs of toxicity

There were no clinical signs observed during the study.

2 .Mortality

There were no mortalities during the course of the study.

B. Body weight and body weight gain

At 8000 ppm, mean body weight was similar to the controls in both sexes on study Day 8 and was slightly reduced by 4% in males and 3% in females on study Day 15 (not statistically significant) in comparison to the controls. This slight effect was attributable to a mean cumulative body weight gain reduced by 13% in males and 12% in females throughout the study (not statistically significant) when compared to the controls.

At 2000 ppm, mean body weight parameters were unaffected by treatment in males, whilst in females a marginal reduction in mean cumulative body weight gain was noted throughout the study (- 12% compared to the controls, not statistically significant).

At 500 ppm, mean body weight parameters were unaffected by treatment in either sex.

C. Food consumption and compound intake

At 8000, 2000 and 500 ppm, mean food consumption was unaffected by treatment in either sex. The mean achieved dose levels of BCS-AA56716 (difluoroacetic acid) at 500, 2000 and 8000 ppm received by the animals during the study were 48, 187 and 745 mg/kg/day in males, respectively, and 51, 201 and 800 mg/kg/day in females, respectively.

D. Clinical pathology

1. Hematology

No treatment-related change was noted at any dose level in either sex. The few differences observed, even if statistically significant, were considered to be incidental in view of the low magnitude of the change and/or absence of dose-effect relationship.

2. Clinical chemistry

Lower mean glucose concentrations were noted at all dose levels in both sexes.

In addition at 8000, 2000 and 500 ppm, slightly higher mean urea concentrations were seen in females (+ 27%, + 25% and + 23%, respectively). Since these changes were not statistically significant and as no relevant variation was noted in creatinine concentrations or at the histological examination, they were considered not to be adverse effects.

Lower mean total bilirubin concentrations (- 45%, p <0.05) were also noted at 8000 and 500 ppm. In the absence of a dose-effect relationship, these variations were considered not to be treatment related.

Table 5.8-06: Significant clinical chemistry changes [Mean ± standard deviation (% change when compared with controls)]

Dose level of BCS-AA56716 (ppm)	0	500	2000	8000
Male				
Glucose (mmol/l)	5.47 ± 0.33	3.62 ± 0.50** (- 34%)	3.25 ± 0.81** (- 41%)	2.50 ± 0.32** (- 54%)
Urea	5.43 ± 0.71	6.46 ± 0.68 (+ 19%)	5.91 ± 0.63 (+ 9%)	5.78 ± 0.71 (+ 6%)
Total bilirubin (µmol/l)	0.9 ± 0.2	0.6 ± 0.2 (- 33%)	0.5 ± 0.2 (- 44%)	0.7 ± 0.2 (- 22%)
Female				
Glucose (mmol/l)	4.89 ± 0.33	2.86 ± 0.42 (- 42%)	2.53 ± 0.56** (-48%)	2.68 ± 0.43** (- 45%)
Urea	5.31 ± 0.38	6.54 ± 0.87 (+ 23%)	6.63 ± 0.59 (+ 25%)	6.77 ± 1.37 (+ 27%)
Total bilirubin (µmol/l)	1.1 ± 0.3	0.6 ± 0.3 * (- 45%)	0.9 ± 0.2 (- 18%)	0.6 ± 02* (- 36%)

* : p <0.05 ** : p <0.01

E. Sacrifice and pathology

1. Organ weights

At 8000 ppm, lower mean terminal body weight was observed in males (- 6%, not statistically significant) when compared to the controls. At 8000 and 2000 ppm, lower mean terminal body weight was observed in females (- 7% and - 6%>, respectively, not statistically significant) when compared to the controls.

2. Gross and histopathology

All changes were considered to be incidental and not treatment-related.

III. Conclusions

In conclusion, the dietary level of 2000 ppm (corresponding to 187 mg/kg body weight/day) was considered to be a NOAEL in males, and the dietary level of 500 ppm (corresponding to 51 mg/kg body weight/day) was considered to be a NOAEL in females.

Oral 90-day toxicity in the rat

Report:	**KIIA 5.8/06, [redacted] P.; 2012**
Title:	BCS-AA56716 (Difluororacetic acid), 90-day toxicity study in the rat by dietary administration
Report No & Document No	SA 10324; M-424611-01-2
Guidelines:	OECD 408 (1998); EEC Directive 2001/59/EC Annex V – Method B.26. (2001); EPA Health Effects Test Guideline (OPPTS 870.3100; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
GLP	Yes (certified laboratory)

Executive Summary

BCS-AA56716 (difluoroacetic acid), a metabolite of the insecticide BYI 02960, (batch number BCOO 5984-5-8, 97.1% w/w purity) was administered continuously via dietary administration to groups of Wistar rats (10/sex/group) at concentrations of 200, 1000 and 6000 ppm (equating to approximately 12.7, 66.2, 380 mg/kg body weight/day in males and 15.6, 78.7, 472 mg/kg body weight/day in females) for at least 90 days. A similarly constituted group of 10 males and 10 females received untreated diet and acted as a control.

Clinical signs were recorded daily, and body weight was measured weekly. Food consumption was measured twice weekly during the first 6 weeks of treatment and weekly thereafter. A detailed physical examination was performed once during the acclimatization phase and weekly throughout the study. All surviving animals were subjected to a neurotoxicity assessment (functional observational battery and spontaneous motor activity) during Week 11 of the study. Ophthalmological examinations were performed on all animals during the acclimatization phase and on all surviving animals of the control and high dose groups during Week 12. Urine samples were collected overnight during the week before necropsy from all surviving animals. Before necropsy a blood sample was collected from the retro-orbital venous plexus of each animal for haematology and clinical chemistry investigations. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically.

Dietary administration of BCS-AA56716 (difluoroacetic acid) to Wistar rats induced no mortalities, no treatment-related clinical signs and no treatment-related changes at the ophthalmological and neurological examinations.

<u>At 6000 ppm</u>

Mean body weight was decreased by up to 7% in males and 9% in females compared to controls (statistically significant on several occasions for females), the effect being progressive throughout the study. At the end of the treatment (study Day 92), the overall mean body weight gain was reduced by 11% in males and 20% in females ($p \leq 0.01$ for females) compared to controls. Mean food consumption was decreased by up to 9% in males (maximal effect observed on study Day 43) and 13% in females (maximal effect observed on study Day 50) throughout the study, compared to controls. The average decrease over weeks 1 to 13 was of 5% in males and 7% in females.

At the hematological evaluation, lower hemoglobin concentration (- 8%, $p \leq 0.01$) and lower mean corpuscular volume (- 5%, $p \leq 0.01$) were observed in females, together with lower mean corpuscular hemoglobin (- 7%, $p \leq 0.01$) and lower hematocrit (- 6%, $p \leq 0.01$), compared to controls. Clinical

chemistry determination revealed lower mean glucose concentrations in both sexes (- 45 and - 53% in males and females, respectively, p ≤0.01), lower total bilirubin concentrations in both sexes (- 56 and - 47% in males and females, respectively, p ≤0.01), slightly higher mean inorganic phosphorus concentrations in both sexes (+ 14%, p ≤0.01 or 0.05) and slightly higher mean urea concentrations in both sexes (+ 18 and + 12% in males and females, respectively, p ≤0.05 in males), compared to controls. Urinalysis revealed higher urinary volumes and lower mean refractive indices in both sexes, lower protein levels in females and lower amounts of usually observed crystals in males, compared to controls. Despite the higher urinary volumes, higher ketone levels were noted in both sexes, in correlation with the low glucose concentrations observed in the serum.
At necropsy, a lower mean terminal body weight was observed in males (- 9%, p≤0.05) and females (- 10%, p≤0.01) when compared to the controls. The few organ weight changes noted were considered to be incidental or attributable to the lower mean terminal body weight. At the macroscopic observation, a few black foci were noted in the glandular part of the stomach in both sexes, in correlation with a few cases of focal glandular erosion/necrosis observed at the microscopic examination.

<u>At 1000 ppm</u>
Mean body weight was decreased by up to 13% in males and 6% in females compared to controls (statistically significant on several occasions). The effect was progressive in both sexes throughout the study, but was slightly more marked in this mid dose male group than at the high dose. At the end of the treatment (study Day 92), the overall mean body weight gain was reduced by 20% in males and 7% in females (p ≤0.01 for males) compared to controls. Mean food consumption was decreased by up to 7% in males (maximal effect observed on study Day 43) and 10% in females (maximal effect observed on study Day 50) throughout the study, compared to controls. The average decrease over weeks 1 to 13 was of 5% in males and 4% in females.
At the hematological evaluation, lower hemoglobin concentration (- 9%, p ≤0.01) and lower mean corpuscular volume (- 6%, p ≤0.01) were observed in females, together with lower mean corpuscular hemoglobin (- 8%, p ≤0.01) and lower hematocrit (- 7%, p ≤0.01). These changes were observed with no clear dose-effect relationship. Clinical chemistry determination revealed lower mean glucose concentrations in both sexes (- 45 and - 49% in males and females, respectively, p ≤0.01), lower total bilirubin concentrations in both sexes (- 52 and - 37% in males and females, respectively, p ≤0.01) and slightly higher mean urea concentrations in both sexes (+ 26 and + 23% in males and females, respectively, p≤0.01) with no clear dose-effect relationshipwhen compared to controls. Urinalysis revealed higher urinary volumes and lower mean refractive indices in both sexes, lower protein levels in females and lower amounts of usually observed crystals in males compared to controls. Despite the higher urinary volumes, higher ketone levels were noted in both sexes, in correlation with the low glucose concentrations observed in the serum.
At necropsy, a lower mean terminal body weight was observed in males (- 15%, p≤0.01) and females (- 7%, p≤0.05) when compared to the controls. The few organ weight changes noted were considered to be incidental or attributable to the lower mean terminal body weight. At the macroscopic observation, a few black foci were noted in the glandular part of the stomach in one or two animals from both sexes (including one control female), in correlation with focal glandular erosion/necrosis observed at the microscopic examination.

<u>At 200 ppm</u>

Treatment-related effects were noted only in the clinical chemistry determination, which revealed lower mean glucose concentrations in both sexes (- 29 and - 27% in males and females, respectively, $p \leq 0.01$), lower total bilirubin concentrations in males (- 27%, $p \leq 0.05$) and slightly higher mean urea concentrations in both sexes (+ 15 and + 14% in males and females, respectively, $p \leq 0.05$). However, these changes are considered not to be adverse effects of the test substance as they do not represent any functional impairment in the test organism since no concomitant change was noted in creatinine concentrations or at the histological examination.

In conclusion, a dose level of 200 ppm (equating to 12.7 and 15.6 mg/kg body weight/day in males and females, respectively) was considered to be a NOAEL in male and female Wistar rats, based on the minor changes noted at the clinical chemistry determination.

I. Materials and Methods

A. Material

1. Test Material:	BCS-AA56716
Description:	Colorless liquid
Lot/Batch:	BCOO 5984-4-11
Purity:	96.7%
CAS:	381-73-7
Stability of test compound:	Stable in rodent diet at 200 and 8000 ppm over a 32-day frozen period followed by 11 days at room temperature
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age:	6 to 7 weeks approximately
Weight at dosing:	183 to 208 g for the males - 150 to 177 g for the females
Source:	[redacted] France
Acclimation period:	6 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum* except at designated time periods
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum* except before urine collection when animals were water fasted overnight
Housing:	Animals were housed five per sex per cage in suspended stainless steel wire mesh cages unless reduced by mortality or isolation

Environmental conditions:

Temperature:	22 ± 2 °C
Humidity:	55 ± 15%
Air changes:	Approximately 10 to 15 changes per hour
Photoperiod:	Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates

January 19 to April 29, 2011 performed at [redacted] France.

2. Animal assignment and treatment

There were 10 animals of each sex per dose group. Animals were assigned to dose groups using a randomization by weight. BCS-AA56716 was administered in the diet for at least 90 days to Wistar rats at the following doses - 200, 1000 and 6000 ppm (equating to approximately 12.7, 66.2, 380 mg/kg body weight/day in males and 15.6, 78.7, 472 mg/kg body weight/day in females). A negative control group received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Table 5.8-07: Study design

Test group	Concentration in diet (ppm)	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	10	10
2	200	12.7	15.6	10	10
3	1 000	66.2	78.8	10	10
4	6 000	380	472	10	10

3. Diet preparation and analysis

The test substance was incorporated into the diet by dry mixing to provide the required dietary concentrations of 200, 1000 or 6000 ppm. There were two preparations for the study. When not in use the diet formulations were stored at approximately -18 °C.

The stability of the test substance in the diet was evaluated in another study at concentrations of 200 and 8000 ppm, where BCS-AA56716 (difluoroacetic acid) was found to be stable in the diet after 11 days at room temperature as well as after a 32-day frozen period followed by 10 days at room temperature: The two preparations of the current study at 200, 1000 and 6000 ppm were used after a maximum storage period of 49 days frozen followed by 8 days at room temperature. The stability of the test substance in the diet was not investigated over this whole duration, but according to the available stability data, it is expected that an extension of the frozen storage period from 32 to 49 days had no impact on the concentration of test material in the diet, since the concentrations measured after 32 days frozen storage followed by 10 days at room temperature were at least equivalent to those measured after 11 days at room temperature.

Table 5.8-08: Stability data of BCS-AA56716 in the diet

% from nominal concentration (ppm)		
	Preparation at 200 ppm	**Preparation at 8000 ppm**
After 11 days at room temperature	101.75% (203.5 ppm)	90.50% (7239.5 ppm)
After a 32-day frozen period followed by 10 days at room temperature	114.25% (228.5 ppm)	95.10% (7607.5 ppm)

Homogeneity of BCS-AA56716 (difluoroacetic acid) in diet for the current study was verified on the first preparation at the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean values obtained from the homogeneity check were taken as measured concentrations. The dietary levels of the test substance were verified for each concentration on each preparation.
All analyses of the substance in diet were performed under the supervision of the Principal Investigator [redacted] France) in compliance with CIT's standard operating procedures.

Results
Homogeneity Analysis
The high- and low-concentration formulations (*i.e.* 200 and 8000 ppm) were found to be homogeneous at initial as coefficients of variation were 3.3 and 5.5%, respectively (in each case below 10%).

Concentration Analysis
The measured concentrations of BCS-AA56716 in the dosage forms remained within a range of - 8.8 to + 9.7%, when compared to the nominal values.

4. Statistics
Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods

1. Clinical signs and mortality
Animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily for all animals during the study. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, duration and recovery of clinical signs were recorded. Cages and cage trays were inspected daily for evidence of ill health such as blood or loose feces.

2. Neurological examinations

During study Week 11, a neurotoxicity assessment was performed for all surviving animals. Each animal was individually tested (the order of animal testing was randomly determined) by the observer who was blind regarding the animal's group assignment. The neurological examination contained all the parameters listed in the study protocol, plus additional assessments (home cage observation, observation during handling and additional physiologic measurements). Spontaneous motor activity was recorded during 60 minutes instead of 90 minutes as initially mentioned in the protocol.

Functional observational battery

Functional Observational Battery included:

- Home cage observation: while the animal was in its home cage, observations were collected regarding posture, piloerection, involuntary motor movements, gait abnormalities, vocalizations or any abnormal behavior
- Observation during handling including ease to remove from cage, reaction to being handled, muscle tone, eyelid, lacrimation, salivation, nasal discharge, staining or any other signs such as alopecia, emaciation, temperature upon touching ("cold to touch")
- Open-field observation: each animal was individually observed in an open field during 2 minutes for piloerection, respiration, arousal, gait abnormalities, posture, involuntary motor movements, stereotypic movements, vocalizations and number of rearings, urine and feces spots
- Reflex and physiologic observations/measurements included:
    - Pupil size
    - Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary constriction by focusing a narrow beam of light in the eyes)
    - Surface righting reflex (by putting the animal on its back and evaluating its ability/rapidity to reassume a normal standing position)
    - Corneal reflex (by touching the medial canthus with a fine object and observing the quick and complete closure of the eyelids)
    - Flexor reflex (by pinching the toes and evaluating the presence/strength of the flexor response of each hindlimb)
    - Auditory startle response (by evaluating the animal response to an auditory stimulus)
    - Tail pinch response (by pinching the tail with a forceps and evaluating the animal reaction)
    - Grip strength: the fore- and hindlimb grip strength of animals was measured quantitatively using a grip strength apparatus equipped with one pull and one push strain gauge (Bioseb, Chaville, France)
    - Landing foot splay: the animal was dropped from approximately 30 cm above a padded surface and hindlimb foot splay was marked, measured and recorded
    - Body weight
    - Rectal temperature.

Spontaneous motor activity

Animals were tested individually using an automated photocell recording apparatus (Imetronic, Bordeaux, France) designed to measure quantitatively spontaneous exploratory locomotor activity in a novel environment. Spontaneous motor activity was recorded during the first 60 minutes with data being collected regular intervals throughout the session.

3. Body weight

Each animal was weighed twice during the acclimatization period, on the first day of test substance administration, then at least weekly throughout the treatment period. Additionally, diet fasted animals were weighed before scheduled necropsy (terminal body weight).

4. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded twice weekly during the first 6 weeks of treatment, then weekly for all animals during the treatment period. From these records the mean daily consumption was calculated. Food spillage was also noted.

The weekly mean achieved dosage intake in mg/kg/day for each week and for Weeks 1 to 13 was calculated for each sex.

5. Ophthalmological examination

During the acclimatization period all animals were subjected to an ophthalmic examination. After instillation of an atropinic agent (Mydriaticum, Merck Sharp and Dohme) each eye was examined by means of an indirect ophthalmoscope. During Week 12 of the treatment period, all surviving animals from control and high dose group were re-examined.

6. Hematology and clinical chemistry

On the day of scheduled sacrifice (study Days 93, 94 or 95), blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus prior to sacrifice. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane (Baxter, Maurepas, France). Blood was collected with EDTA for hematology, with clot activator for serum clinical chemistry and with sodium citrate for coagulation parameters.

Hematology

The following haematology parameters were assayed using an Advia 120 (Siemens, Eragny, France): red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count. A blood smear was prepared and stained with May-Grünwald-Giemsa method.. It was examined when results of Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL Elite Pro (Instrumentation Laboratory, Paris, France).

Clinical chemistry

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus concentrations and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed on plasma samples, total protein and albumin concentrations were assayed on serum samples using an Advia 1650 (Siemens, Eragny, France). Globulin and albumin/globulin ratio values were calculated.

7. Urinalysis

On the morning of study Days 87 or 88 overnight urine samples were collected from all animals in all groups. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Food and water were not accessible during urine collection.
Any significant change in the general appearance of the urine was recorded. Urine samples were weighed to determine urinary volume. pH was assayed using a Clinitek 500 and Multistix dipsticks (Siemens, Eragny, France). Urinary refractive index was measured using a RFM320 refractometer (Bioblock Scientific, Illkirch, France).
Glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen were assayed using a Clinitek 500 and Multistix dipsticks (Siemens, Eragny, France).

Microscopic examination of the urinary sediment was performed after centrifugation of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was graded.

8. Sacrifice and pathology

Necropsy procedure

On study Days 93, 94 or 95, all animals from all groups were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day of sacrifice. Animals were diet fasted overnight prior to sacrifice.
All animals were necropsied. The necropsy included the examination of all orifices, major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically.

Histopathology

On study days 92, 93 or 94, a complete necropsy was performed on all surviving animals. Animals were deeply anaesthetized by inhalation of Isoflurane, and then exsanguinated before necropsy. All animals were fasted prior to scheduled sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically.
Adrenal gland, brain, epididymides, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.
The following organs or tissues were sampled: adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, oesophagus, exorbital (lachrymal) gland, eye and optic nerve, harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina.
A bone marrow smear was prepared from femur, stained with May-Grünwald Giemsa, but not examined. Tissues samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, harderian gland, epididymis and testis that were fixed in Davidson's fixative.

All of the above samples (except exorbital lachrymal gland, larynx/pharynx and nasal cavities) from all animals in all groups were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared from all organs and tissue samples from all the animals in the control and high dose groups.
Additionally, sections from liver, kidney, lung, stomach and thyroid gland were prepared for all the animals in all intermediate dose groups.
All the tissues processed were examined.
Initial examinations were performed by the Study Pathologist. Following the initial examination, an in-house pathologist, undertook an independent « peer-review » of representative slides and diagnoses according to standardized operating procedures. The diagnoses presented here represent the consensus opinion of the two pathologists.

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 course of the study.

3. Neurological examinations

Functional observational battery (FOB) observations

Up to 6000 ppm included, no treatment-related neurobehavioral changes were recorded during the FOB observations in home cage, during handling or in open field in either sex between any treated groups compared to controls. The few changes observed at 6000 ppm (repetitive licking of lips and stereotypic grooming observed in 4/10 males and 2/10 females, respectively) were considered to be incidental since they were within the normal range of variation and/or had no corroborating signs at the neurological examination.
No treatment-related changes were observed in any of the reflexes and responses evaluated in either sex in any of the treated groups compared to controls. At 6000 ppm, 3/10 males had weak tail pinch response, however in the absence of any corroborating signs, this change was considered to be incidental.

Grip strength, landing foot splay, rectal temperature and body weight

No relevant treatment-related changes were observed in grip strength, landing foot splay and rectal temperature parameters in either sex at any dose level compared to controls.
The statistically lower mean forelimb grip strength ($p \leq 0.01$) observed in females at 1000 ppm was considered to be incidental since it was seen without dose-relationship and had no corroborative effect (*i.e.* effect on hindlimb grip strength). Lower mean body weight was observed in males at 1000 ppm and in females at 6000 ppm ($p \leq 0.01$) which correlates well with the effects on mean body weight seen throughout the study

Spontaneous motor activity

No changes were recorded in overall mean spontaneous motor activity in either sex at any dose levels compared to controls. In addition, the general pattern of motor activity within the test session (*e.g.* habituation) in test groups was similar to the control group, with no evidence of a treatment-related effect at any dose level.

B. Body weight and weight gain

At 6000 ppm, mean body weight was decreased by up to 7% in males and 9% in females compared to controls (statistically significant on several occasions for females), the effect being progressive throughout the study. At the end of the treatment (study Day 92), the overall mean body weight gain was reduced by 11% in males and 20% in females ($p \leq 0.01$ for females) compared to controls.
At 1000 ppm, mean body weight was decreased by up to 13% in males and 6% in females compared to controls (statistically significant on several occasions). The effect was progressive in both sexes throughout the study, but was slightly more marked in the mid dose male group than at the high dose. At the end of the treatment (study Day 92), the overall mean body weight gain was reduced by 20% in males and 9% in females ($p \leq 0.01$ for males) compared to controls.
There were no treatment-related effects on mean body weight parameters in both sexes at 200 ppm compared to controls.

Table 5.8-09: Mean body weights (BW) and cumulative body weight gains (BWG)

Dosage level of BCS-AA56716 (ppm)	**0**	**200**	**1000**	**6000**
Male				
Initial BW (Day 1) (%C)	195	196 (101)	195 (100)	196 (101)
BW Week 2 (Day 8) (%C)	257	259 (101)	247 (96)	255 (99)
BW Week 4 (Day 29) (%C)	380	379 (100)	348 (91)**	364 (96)
BW Week 8 (Day 57) (%C)	465	472 (102)	408 (88)**	435 (94)
Final BW Week 13 (Day 92) (%C)	**512**	**531 (104)**	**450 (88)****	**479 (94)**
BWG Weeks 1-4 (Days 1 to 29) (%C)	186	183 (97)	153 (82)**	168 (90)
BWG Weeks 1-8 (Days 1 to 57) (%C)	270	276 (102)	213 (79)**	239 (88)
Overall BWG Weeks 1-13 (Days 1-92) (%C)	**317**	**335 (105)**	**255 (80)****	**283 (89)**
Female				
Initial BW (Day 1) (%C)	**162**	**164 (101)**	**161 (100)**	**162 (100)**
BW Week 1 (Day 8) (%C)	188	189 (101)	188 (100)	184 (98)
BW Week 4 (Day 29) (%C)	240	242 (101)	235 (98)	225 (94)**
BW Week 8 (Day 57) (%C)	274	276 (101)	260 (95)	255 (93)**
Final BW Week 13 (Day 92) (%C)	**295**	**295 (100)**	**283 (96)**	**268 (91)****
BWG Weeks 1-4 (Days 1 to 29) (%C)	78	78 (100)	74 (94)	63 (81)**
BWG Weeks 1-8 (Days 1 to 57) (%C)	112	112 (100)	99 (88)*	93 (83)**
Overall BWG Weeks 1-13 (Days 1-92) (%C)	**133**	**131 (99)**	**121 (91)**	**106 (80)****

% C : % *vs.* control, calculated on raw data
* : Statistically different ($p \leq 0.05$) from the control
** : Statistically different ($p \leq 0.01$) from the control

C. Food consumption

At 6000 ppm, mean food consumption was decreased by up to 9% in males (maximal effect observed on study Day 43) and 13% in females (maximal effect observed on study Day 50) throughout the study compared to controls. The average decrease over weeks 1 to 13 was 5% in males and 7% in females.
At 1000 ppm, mean food consumption was decreased by up to 7% in males (maximal effect observed on study Day 43) and 10% in females (maximal effect observed on study Day 50) throughout the study compared to controls. The average decrease over weeks 1 to 13 was 5% in males and 4% in females.
Mean food consumption was unaffected by the treatment at 200 ppm in both sexes throughout the study.

D. Achieved dosages

The mean achieved dose levels of BCS-AA56716 (difluoroacetic acid) expressed in mg/kg/day received by the animals during the study were as follows:

Table 5.8-10: Mean achieved dietary intake of BCS-AA56716 (Weeks 1 – 13)

Diet concentration (ppm)	Male mg/kg/day	Female mg/kg/day
200	12.7	15.6
1000	66.2	78.7
6000	380	472

E. Ophthalmological examination

No treatment-related ocular abnormalities were observed at ophthalmoscopic examination.

F. Blood analysis

1. Haematological findings

When compared to controls, lower hemoglobin concentration and lower mean corpuscular volume were observed at 6000 and 1000 ppm in females only. As a consequence, lower mean corpuscular hemoglobin and lower hematocrit were also noted. These changes were observed with no clear dose-effect relationship.
No variation was noted in erythrocyte and reticulocyte counts.

The statistically significant lower mean corpuscular hemoglobin observed at 200 ppm was not considered to be biologically relevant in view of its low magnitude.
The few other differences observed, even if statistically significant, were considered to be incidental.
No treatment-related change was noted in males at any dose level and in females at 200 ppm.

Table 5.8-11: Hematological changes in females - Mean ± standard deviation (% change when compared to controls)

BCS-AA56716 Dose level (ppm)	0	200	1000	6000
Hemoglobin concentration (g/dL)	15.89 ± 0.608	15.43 ± 0.387 (- 3%)	14.43 ± 0.670** (- 9%)	14.60 ± 0.440** (- 8%)
Mean corpuscular volume (fL)	54.6 ± 1.25	53.6 ± 1.28 (- 2%)	51.3 ± 0.80** (- 6%)	52.1 ± 0.85** (- 5%)
Mean corpuscular hemoglobin (pg)	17.76 ± 0.519	17.23 ± 0.427* (- 3%)	16.35 ± 0.387** (- 8%)	16.58 ± 0.391** (- 7%)
Hematocrit (L/L)	0.4883 ± 0.0153	0.4797 ± 0.0107 (- 2%)	0.4529 ± 0.0206** (- 7%)	0.4596 ± 0.0158** (- 6%)

* : $p \leq 0.05$ ** : $p \leq 0.01$

2. Clinical chemistry findings

Statistically significantly lower mean glucose concentrations were noted at all dose levels in both sexes.
Statistically significantly lower mean total bilirubin concentrations were observed at 6000 and 1000 ppm in both sexes and at 200 ppm in males only. However, these changes are considered not to be adverse effects of the test substance as they do not represent any functional impairment in the test organism.
Slightly higher mean inorganic phosphorus concentrations were noted at 6000 ppm in males (+ 14%, $p \leq 0.01$) and in females (+ 14%, $p \leq 0.05$).
Slightly higher mean urea concentrations were seen at all dose levels in both sexes. There was no dose-effect relationship and as no concomitant change was noted in creatinine concentrations or at the histological examination, these differences were considered not to be adverse effects.
No other treatment-related change was noted. The few other differences observed, even if statistically significant, were considered to be incidental and/or not biologically relevant.

Table 5.8-12: Clinical chemistry changes - Mean ± standard deviation (% change when compared to controls)

BCS-AA56716 Dosage level (ppm)		0	200	1000	6000
Glucose concentration (mmol/L)	Male	5.677 ± 0.535	4.003 ± 0.556** (- 29%)	3.141 ± 0.393** (- 45%)	3.123 ± 0.245** (- 45%)
	Female	6.036 ± 0.490	4.428 ± 0.544** (- 27%)	3.065 ± 0.587** (- 49%)	2.815 ± 0.321** (- 53%)
Total bilirubin concentration (µmol/L)	Male	1.47 ± 0.356	1.07 ± 0.442* (- 27%)	0.70 ± 0.258** (- 52%)	0.65 ± 0.295** (- 56%)
	Female	2.45 ± 0.784	2.16 ± 0.521 (- 12%)	1.55 ± 0.617** (- 37%)	1.30 ± 0.343** (- 47%)
Urea concentration (mmol/L)	Male	4.831 ± 0.522	5.569 ± 0.602 * (+ 15%)	6.076 ± 0.811 ** (+ 26%)	5.702 ± 0.569 * (+ 18%)
	Female	5.509 ± 0.651	6.253 ± 0.378 * (+ 14%)	6.764 ± 0.737 ** (+ 23%)	6.165 ± 0.600 (+ 12%)

G. Urinalysis

Treatment-related changes were observed in males at all dietary levels and in females at 6000 and 1000 ppm compared to controls. The parameters affected in these groups are listed hereafter:
Mean urinary volumes were higher in the aforementioned treated groups. As a consequence, lower mean refractive indices were noted in these groups, lower protein levels were seen in females and lower amounts of usually observed crystals were noted in the 6000 and 1000 ppm male groups.

Table 5.8-13: Urinary volume (mL) - Mean ± standard deviation (% change when compared to controls)

BCS-AA56716 Dose level (ppm)	0	200	1000	6000
Male	3.50 ± 1.084	7.43 ± 1.730 (+ 112%)	11.91 ± 3.731 *** (+ 240%)	12.80 ± 4.508 *** (+ 266%)
Female	1.66 ± 1.727	2.13 ± 1.286 (+ 28%)	3.41 ± 1.559 (+ 105%)	4.24 ± 1.620 ** (+ 155%)

** : p ≤0.01 *** : p ≤0.001

Despite the higher urinary volumes, higher ketone levels were noted in the same treated groups. At 200 ppm in males the difference from the controls was marginal. In the other groups it was marked and dose-related in females. This change was correlated with the low glucose concentrations observed in the serum.

Table 5.8-14: Urinary ketone level - (number of urine samples observed per grade)

BCS-AA56716 Dose level (ppm)		Control	200	1000	6000
Male	Grade 0	0	0	0	0
	1	7	2	0	0
	2	3	8	0	0
	3	0	0	0	0
	4	0	0	10	10
	total	10	10	10	10
Female	Grade 0	6	6	0	0
	1	1	2	0	0
	2	0	0	5	0
	3	0	0	4	7
	4	0	0	0	2
	total	7	8	9	9

A statistically significantly lower urinary pH was observed in males at 6000 ppm, but it was considered not to be biologically relevant.

H. Sacrifice and pathology

1. Terminal body weight and organ weights

At 6000 and 1000 ppm, mean terminal body weight was lower in males (- 9%, p <0.05 and - 15%, p <0.01, respectively) and in females (- 10%, p <0.01 and - 7%, p <0.05, respectively), when compared to controls.

The few organ weight changes noted were considered to be incidental or attributable to the lower mean terminal body weight.

2. Gross Pathology

At 6000 and 1000 ppm, a few black foci were noted in the glandular part of the stomach in both sexes. This change was considered treatment-related and mostly correlated with focal glandular erosion/necrosis observed at the microscopic examination. All other changes were considered as incidental and not treatment-related.

Table 5.8-15: Incidence of macroscopic changes in the stomach

Sex	Male				Female			
BCS-AA56716 dose level (ppm)	0	200	1000	6000	0	200	1000	6000
Focus(i), black	0/10	0/10	1/10	3/10	1/10	0/10	2/10	3/10

3. Microscopic Pathology

At 6000 and 1000 ppm, focal glandular erosion/necrosis was noted in the stomach in both sexes. These changes were considered to be treatment-related. All other changes were considered as incidental and not treatment-related.

Table 5.8-16: Incidence and severity of microscopic changes in the stomach

Sex	Male				Female			
BCS-AA56716 dose level (ppm)	**0**	**200**	**1000**	**6000**	**0**	**200**	**1000**	**6000**
Number of animals examined	10	10	9	10	10	10	10	10
Glandular erosion/necrosis - focal								
Minimal	0	0	1	1	0	0	0	0
Slight	0	0	0	1	0	0	0	1
Moderate	0	0	0	0	0	0	1	0
Total	**0**	**0**	**1**	**2**	**0**	**0**	**1**	**1**

III. Conclusions

In conclusion, a dose level of 200 ppm (equating to 12.7 and 15.6 mg/kg body weight/day in males and females, respectively) was considered to be a NOAEL in male and female Wistar rats, based on the minor changes noted at the clinical chemistry determination.

BYI 02960-difluoroethylamino-furanone

In vitro *genotoxicity - Bacterial assay for gene mutation*

Report:	**KIIA 5.8/07, [redacted] A.; 2011**
Title:	Salmonella Typhimurium, Reverse mutation assay with BYI 02960-Difluoroethyl-amino-furanone
Report No & Document No	1399701 M-409728-01-2
Guidelines:	OECD 471 (1997); EEC Directive N° 440/2008 B13/14 (2008); EPA Health Effects Test Guideline (OPPTS 870.5100; 1998)
GLP	Yes (certified laboratory) except that no analytical analyses were performed during the study

Executive Summary

This study was performed to investigate the potential of BYI 02960-difluoroethyl-amino-furanone, metabolite of BYI 02960 (batch N° NLI 8671-6-1, 98.5% of purity) to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.
The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations: Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate; Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate.
The plates incubated with the test item showed normal background growth in all strains with or without S9 mix in both experiments up to 5000 µg/plate.
No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in all strains with or without S9 mix in both experiments. .No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BYI 02960-difluoroethyl-amino-furanone at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.
Therefore, BYI 02960-difluoroethyl-amino-furanone is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960-difluoroethyl-amino-furanone
Description:	White powder
Lot/Batch:	NLL 8671-6-1
Purity:	98.5%
CAS:	1134834-71-1
Stability of test compound:	No analysis performed during the study

2. Control materials:

Negative: Culture medium
Solvent: DMSO
Positive: Sodium azide (Serva) for TA 1535 and TA 100 at 10 µg/plate,
4-Nitro-1,2-phenylene diamine (Sigma) for TA 1537 at 50 µg/plate and TA 98 at 10 µg/plate,
Methyl methane sulfonate (Sigma) for TA 102 at 3.0 µL/plate,
2-Aminoanthracene (Sigma Aldrich) for the activating effect of the S9 mix in all strains at 2.5 µg/plate for all strains except for TA 102 at 10.0 µg/plate

3. Test organisms:

Species: Salmonella typhimurium LT2 mutants
Strain: Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102
Source: Strains obtained from [redacted], Germany)

4. Test compound concentrations:

Experiment I: First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate

Experiment II: For TA 1535, TA 1537, TA98, TA100 and TA 102 with or without S9 mix: 33, 100, 333, 1000, 2500 and 5000 µg/tube

B. Study Design and methods

The experimental phase of the study was performed between March 31 to April 14, 2011 at [redacted], Germany.

The Salmonella/microsome test is a screening method which detects point mutations caused by chemical agents *in vitro*. Auxotrophic mutants of Salmonella typhimurium are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

1. Plate incorporation assay (experiment I)

DMSO (0.1 mL) containing BYI 02960-difluoroethyl-amino-furanone or controls were added to glass vessels with 0.1 mL of bacterial cultures grown overnight, 0.5 mL of S9 mix or buffer and 2 mL of soft agar. The mixture was placed in a waterbath at 45 °C for 30 seconds, shaken and overlaid onto Petri dishes containing solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were scored using the Petri Viewer Mk2. Three plates were used, both with and without S9 mix, for each strain and dose. The doses for the first trial were routinely determined on the basis of a standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses. If less than three doses were used for assessment, at least two repeats were performed.

2. Pre-incubation assay (experiment II)

An independent repeat was performed as pre-incubation of the previously described mixture in a water bath at 37 °C for 60 minutes. At the end of the preincubation period, 2 mL of molten soft agar were added to the tubes, the content mixed and plated onto Petri dishes with solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were also scored using an automated colony counter.

3. Assessment criteria

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100 and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed. A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration. An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and discussion

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BYI 02960-difluoroethyl-amino-furanone at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

The lower limit of the laboratory's historical control range was not quite reached in the untreated control of strain TA 102 with metabolic activation in the pre-experiment/*experiment I*. Since this deviation is

rather small it is judged to be based on biologically irrelevant fluctuations in the number of colonies and has no impact on the outcome of the study.
In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

III. Conclusions

No indication of mutagenic effects of BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960) could be found at assessable doses of up to 5000 µg/plate in any of the Salmonella typhimurium strains used in the assay.

In vitro *genotoxicity - Test for clastogenicity in mammalian cells*

Report:	**KIIA 5.8/08, [redacted] C, 2011**
Title:	BYI 02960-difluoroethyl-amino-furanone (Metabolite of BYI 02960): *In vitro* chromosome aberration test with Chinese Hamster V79 cells
Report No & Document No	1399703 M-420108-01-2
Guidelines:	OECD 473 (1997); EEC Directive 440/2008 Method B10; EPA Health Effects Test Guideline (OPPTS 870.5375; 1998)
GLP	Yes (certified laboratory)

Executive Summary

In this *in vitro* assessment of the clastogenic potential of BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960) (batch NLL 8671-6-1, 98.5% of purity), Chinese Hamster V79 cells were exposed to BYI 02960-difluoroethyl-amino-furanone at 6.4, 12.8, 25.6, 51.1, 102.3, 204.5, 409, 818 and 1636 µg/mL (1636 µg/mL = 10 mM), diluted in dimethyl sulphoxide (DMSO). For each dose level, duplicate cultures were used in both the presence and absence of a metabolic activation system (S9 mix). DMSO was also used as a negative control. Ethylmethane sulfonate, which produces crosslinks in the DNA, and cyclophosphamide, which induces chromosomal damage after metabolic activation, were used as positive controls. After 4 hours treatment, the medium was changed and the cells were harvested 14 hours later. No additional experiment was performed. Colcemid was added to each flask two hours prior to harvest to arrest the cells in a metaphase-like stage of mitosis.

In the absence and presence of S9 mix neither test item precipitation nor relevant cytotoxicity were observed up to the highest required concentration.
Clastogenicity was observed at concentrations from 12.8 to 102.3 and from 409 to 1636 mg/mL without metabolic activation without showing a clear dose-dependent effect. In the presence of S9 mix no clastogenicity was observed.
Appropriate mutagens were used as positive controls. They induced statistically significant increases ($p < 0.05$) in cells with structural chromosome aberrations.

In conclusion, it can be stated that biologically relevant increases of chromosomal aberrations were observed and BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960) is classified as clastogenic in absence of metabolic activation.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960-difluoroethyl-amino-furanone
Description:	White powder
Lot/Batch:	NLL 8671-6-1
Purity:	98.5%
CAS:	1134834-71-1
Stability of test compound:	No analysis performed during the study
2. Control materials:	Negative: Culture medium
	Solvent: Deionized water for BYI 02960-difluoroethyl-amino-furanone
	Positive: Ethylmethane sulfonate (Acros organics, Belgium) without S9 mix at 1000 µg/Cyclophosphamide (Sigma-Aldrich, Germany) with S9 mix at 1.4 µg/mL
3. Test organisms:	
Cell line:	Chinese hamster V79 lung cells
Source:	Cells supplied by [redacted] Germany
Culture condition:	Incubation performed at 37 °C in a humidified atmosphere with about 1.5% CO_2
4. Test compound concentrations:	In experiment I, exposure period 4 hours with or without S9 mix, BCS-AA56716 was used at 6.4, 12.8, 25.6, 51.5, 102.3, 204.5, 409, 818 and 1636 µg/mL

B. Study design and methods

The experimental phase of the study was performed from March 16 to April 15, 2011 at [redacted] Germany.

The *in vitro* cytogenetic test is a mutagenicity test system for the detection of chromosome aberrations in cultured mammalian cells. The test is designed to detect structural aberrations (chromatid and chromosome aberrations) in cells at their first post-treatment mitosis.

1. Determination of cytotoxicity

With respect to the molecular weight of the test item 1636 µg/mL of BYI 02960-difluoroethyl-amino-furanone (approx. 10 mM) was applied as top concentration for treatment of the cultures in the pre-test.

Test item concentrations between 6.4 and 1636 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.
Due to the results obtained in Experiment I, no further experiment was performed. No cytotoxicity was observed up to the highest applied concentration.

2. Seeding of the cultures
Thawed stock cultures were propagated at 37° C in 80 cm^2 plastic flasks. About 5 x 10^6 cells per flask were seeded in 15 mL of MEM (minimal essential medium) containing Hank's salts and 10 % (v/v) fetal bovine serum (FBS). Additionally, the medium was supplemented with neomycin (5 µg/mL), Hepes (25 mM) and amphotericin B (2.5 µg/mL). The cells were subcultured twice a week. The cell cultures were incubated at 37° C in a humidified atmosphere with 1.5 % carbon dioxide (98.5% air).

Exponentially growing stock cultures more than 50 % confluent were rinsed with Ca-Mg-free salt solution containing 8000 mg/L NaCl, 200 mg/L KCl, 200 mg/L KH_2PO4 and 50 mg/L Na_2HPO_4. Afterwards the cells were treated with trypsin-EDTA solution at 37° C for approx. 5 minutes. Then, by adding complete culture medium including 10% (v/v) FBS the enzymatic treatment was stopped and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.5% (w/v) in Ca-Mg-free salt solution. The cells were seeded into Quadriperm dishes, which contained microscopic slides. Into each chamber 1 x 10^4 - 6 x 10^4 cells were seeded with regard to the preparation time.

3. Treatment protocol
Exposure period 4 hours
The culture medium of exponentially growing cell cultures was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium were added. Concurrent solvent and positive controls were performed. After 4 hours the cultures were washed twice with saline. The cells were then cultured in complete medium containing 10 % (v/v) FBS for the remaining culture time of 14 hours.

4. Preparation of the cultures
Colcemid was added to the culture medium (0.2 µg/mL) 15.5 hours after the start of the treatment. The cells were treated, 2.5 hours later, on the slides in the chambers with hypotonic solution (0.4 % KCl) for 20 min at 37° C. After incubation in the hypotonic solution the cells were fixed with a mixture of methanol and glacial acetic acid (3 : 1 parts, respectively). After preparation the cells were stained with Giemsa and labelled with a computer-generated random code to prevent scorer bias.

5. Evaluation of cell numbers
The evaluation of cytotoxicity indicated by reduced cell numbers was made after the preparation of the cultures on spread slides. The cell numbers were determined microscopically by counting 10 defined fields per coded slide. The cell number of the treatment groups is given in percentage compared to the respective solvent control.

6. Analysis of metaphase cells

Evaluation of the cultures was performed using NIKON microscopes with 100x objectives. Breaks, fragments, deletions, exchanges, and chromosome disintegrations were recorded as structural chromosome aberrations. Gaps were recorded as well but not included in the calculation of the aberration rates. At least 100 well spread metaphases per culture were evaluated for cytogenetic damage on coded slides, except for the positive control without metabolic activation, where only 50 metaphases were evaluated. Only metaphases with characteristic chromosome numbers of 22 ± 1 were included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined.

7. Evaluation criteria

A test item was classified as non-clastogenic if:

- The number of induced structural chromosome aberrations in all evaluated dose groups was in the range of the laboratory's historical control data,

and/or

- no significant increase of the number of structural chromosome aberrations was observed.

A test item is classified as dastogenic if:

- The number of induced structural chromosome aberrations is not in the range of the laboratory's historical control data,

and

- either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

Statistical significance was confirmed by means of the Fisher's exact test ($p < 0.05$). However, both biological and statistical significance should be considered together, if the criteria mentioned above for the test item were not clearly met, the classification with regard to the historical data and the biological relevance was discussed and/or a confirmatory experiment was performed.

Although the inclusion of the structural chromosome aberrations was the purpose of this study, it was important to include the polyploids and endoreduplications.

8. Assessment criteria

The chromosome aberration test was considered acceptable if it met the following criteria:

- The number of structural aberrations found in the solvent controls fell within the range of the laboratory's historical control data.
- The positive control substances produced significant increases in the number of cells with structural chromosome aberrations which were within the range of the laboratory's historical control data.

II. Results and discussion

The test item BYI 02960-dfluoroethyl-amino-furanone (metabolite of BYI 02960), dissolved in deionized water, was assessed for its potential to induce structural chromosome aberrations in V79 cells of the Chinese hamster *in vitro* in the absence and presence of metabolic activation by S9 mix. According to the OECD guideline only one experiment was performed, since the test item was considered to be mutagenic after the first experiment. In this main experiment the exposure period was 4 hours with and without S9 mix. The chromosomes were prepared 18 hours after the start of treatment with the test item. Two parallel cultures were set up. 100 metaphases per culture were evaluated for structural chromosome aberrations, except for the positive control without metabolic activation, where only 50 metaphases were evaluated.

Neither precipitation nor relevant influence of the test item on pH value or osmolarity was observed. No cytotoxicity indicated by reduced cell numbers or mitotic indices were observed after 4 hours treatment with the test item up to the maximum applied dose group.

In the absence of S9 mix the aberration rates were statistically and significantly increased in all dose groups (6.4 to 1636 µg/mL): 4.0 to 8.5 % aberrant cells, excluding gaps were determined in comparison to the corresponding controls showing 1.0% aberrant cells, excluding gaps. The values of historical control data for chromosomal aberration showed a range from 0.0 to 4.0% aberrant cells, excluding gaps. Therefore, the two test item concentrations showing values of 4.0% aberrant cells, excluding gaps were not biologically relevant. However, all other values clearly exceeded the laboratory's historical data range without showing a clear dose-dependency. Also the number of cells carrying exchanges was distinctly increased after treatment with the test item in the absence of metabolic activation (2.0 to 5.0%) as compared to the solvent control (0.5%).

In the presence of S9 mix the aberration rates were lower than the respective solvent control and remained well within the laboratory's historical data range.

Single increased values of polyploidy metaphases (5.0 to 9.3%) and endomitotic cells (0.9 to 1.7%) were observed in different dose groups as compared to the rates of the solvent controls (4.4 and 0.5%). Either EMS (1000 µg/mL) or CPA (1.4 µg/mL) were used as positive controls and showed distinct increases in the number of cells with structural chromosome aberrations.

Table 5.8-17: Summary of the results of the chromosomal aberration study with BYI 02960-dfluoroethyl-amino-furanone

Test item concentration in µg/mL	Endomitotic cells in %	Polyploid cells in %	Cell number in % of control	Mitotic indices in % of control	Aberrant cells in %		
					Including gaps £	Excluding gaps £	With exchanges
Exposure period 4 hours without S9							
Deionized water 10%	0.5	4.4	100.0	100.0	1.0	1.0	0.5
EMS 1000 µg/mL $	0.0	3.8	n.t.	94.9	39.0	39.0 ***	36.0
6.4	0.6	4.3	88.6	121.7	4.0	4.0 *	2.0
12.8	0.3	5.2	97.2	112.1	4.5	4.5 *	2.0
25.6	0.6	5.0	95.0	116.5	9.0	8.5 ***	5.0
51.5	0.3	4.0	105.7	99.6	8.5	8.0 ***	2.0
102.3	1.0	6.4	96.6	92.6	5.5	5.5 **	3.0
204.5	1.7	9.3	89.3	117.3	4.5	4.0 *	2.5
409.0	0.5	8.9	102.7	86.0	5.0	4.5 *	3.5
818.0	0.4	8.1	91.1	87.1	7.5	7.5 ***	4.5
1636.0	0.9	6.8	85.8	88.6	8.0	7.5 ***	4.0
Exposure period 4 hours with S9							
Deionized water 10%	0.3	4.5	100.0	100.0	3.0	3.0	0.5
CPA 1.4 µg/mL	0.0	3.9	n.t.	65.0	12.5	12.0 ***	2.5
40.9.0	0.0	2.7	94.0	106.7	0.5	0.5	0.0
818.0	0.4	4.5	88.0	112.3	1.5	1.0	0.0
1636.0	0.1	3.5	103.6	97.9	2.0	2.0	1.0

£ : inclusive cells carrying exchanges $: Evaluation of 50 metaphases per culture
µ : evaluation of 200 metaphases per culture n.t. : not tested
* : p ≤ 0.05 ** : p ≤ 0.01 *** : p ≤ 0.001

III. Conclusions

In conclusion, it can be stated that under the experimental conditions reported, the test item BYI 02960-difluoroaethyl-amino-furanone (metabolite of BYI 02960) induced structural chromosome aberrations in V79 cells (Chinese hamster cell line) in the absence of metabolic activation at all tested doses.

In vitro *genotoxicity - Test for clastogenicity in mammalian cells*

Report:	**KIIA 5.8/09, [redacted] H.-E.; 2011**
Title:	BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960), Gene mutation assay in Chinese Hamster V79 cells *in vitro* (V79/HPRT)
Report No & Document No	1399702 M-420095-01-2
Guidelines:	OECD 476 (1997); EEC Directive 440/2008 Method B17 (2008); EPA Health Effects Test Guideline (OPPTS 870.5300; 1998)
GLP	Yes (certified laboratory)

Executive Summary

The study was performed to investigate the potential of BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960) (Batch N° NLL 8671-6-1, 98.5% of purity) to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster.
The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours at concentrations ranging from 51.3 to 1640 µg/mL. The second experiment was performed with a treatment time of 4 hours with metabolic activation and 24 hours without metabolic activation at concentrations ranging from 51.3 to 1640 µg/mL.

No substantial and reproducible dose dependent increase of the mutation frequency was observed in either of the main experiments.
Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies and thus, showed the sensitivity of the test item and the activity of the metabolic activation system.
In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells. Therefore, BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960) is considered to be non-mutagenic in this HPRT assay.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960-difluoroethyl-amino-furanone

Description:	White powder
Lot/Batch:	NLL 8671-6-1
Purity:	98.5%
CAS:	1134834-71-1
Stability of test compound:	No analysis performed during the study

2. Control materials:

Negative: Culture medium: Minimal essential medium supplemented with 1% of Neomycin and 10% foetal calf serum (FCS)

Solvent: Deionized water (10%) for the test item, DMSO for Dimethylbenzanthracene not exceeding 0.5% (v/v) in the culture medium. No solvent needed for ethyl methanesulfonate as it is a liquid.

Positive: Ethyl methanesulfonate (EMS), a directly alkylating agent, used at a final concentration of 150 µg/mL (1.2 mM) in non-activation trials.
Dimethylbenzanthracene (DMBA), promutagen requiring a metabolic activation, used at a final concentration of 1.1 µg/mL for trials with S9 mix.

3. Test organisms:

Cell line: Chinese hamster V79 lung cells

Source: Cells supplied by [redacted], Technical [redacted], Germany

Culture condition: Incubation performed at 37 °C in a humidified atmosphere with about 4.5% CO_2.

4. Test compound concentrations: BYI 02960-difluoroethyl-amino-furanone was used from 51.3 to 1640 µg/mL

5. Metabolic activation: The S9 fraction was isolated from the livers of Phenobarbital/β-naphthoflavone induced male Wistar rats. The protein concentration of the S9 preparation was 35.2 mg/mL in the pre-experiment and experiment I and 32.6 mg/mL in experiment II.

B. Study Design and methods

The experimental phase of the study was performed from March 17 to July 08, 2011 at [redacted] Germany.

The selection of V79 forward mutations is based on the resistance of induced mutants to the purine analogue 6-thioguanine (6-TG). This resistance is a result of a mutation at the X-chromosome-linked HPRT locus rendering the cells unable to use 6-TG for DNA synthesis. Therefore, cell colonies formed in the presence of 6-TG are considered to represent mutants at the HPRT gene.

1. Determination of cytotoxicity

The general culture conditions and experimental conditions in this pre-test were the same as described for the mutagenicity experiment below. In this pre-test the colony forming ability of approximately 500 single cells (duplicate cultures per concentration level) after treatment with the test item was observed and compared to the controls.

Toxicity of the test item was indicated by a reduction of the cloning efficiency (CE).

2. Treatment protocol

Thawed stock cultures were propagated at 37 °C in 80 cm^2 plastic flasks. About 5 x 10^5 cells were seeded into each flask with 15 mL of MEM (minimal essential medium) containing Hank's salts, neomycin (5 µg/mL) and Amphotericin B (1%). The cells were sub-cultured twice weekly. The cell cultures were incubated at 37 °C in a 1.5% carbon dioxide atmosphere (98.5% air). For the selection of mutant cells the complete medium was supplemented with 11 pg/mL 6-thioguanine.
Three days after sub-cultivation stock cultures were trypsinized at 37 °C for 5 minutes. Then the enzymatic digestion was stopped by adding complete culture medium with 10% fetal calf serum and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.2% in Ca-Mg-free salt solution.
The cell suspension was seeded into plastic culture flasks. Approximately 4.5 x 10^6 (single culture) and 5 x 10^2 cells (in duplicate) were seeded in MEM with 10 % FBS (complete medium) for the determination of mutation rate and toxicity, respectively.
After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps. In the second experiment the cells were exposed to the test item for 24 hours in complete medium, supplemented with 10% FBS, in the absence of metabolic activation.

Three days (experiment I) or four days (experiment II) after treatment 1.5 x 10^6 cells per experimental point were sub-cultivated in 175 cm^2 flasks containing 30 mL medium. Following the expression time of 7 days five 80 cm^2 cell culture flasks were seeded with about 3 - 5x10^5 cells each in medium containing 6-TG. Two additional 25 cm^2 flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% $C0_2$ for about 8 days. The colonies were stained with 10 % methylene blue in 0.01% KOH solution. The stained colonies with more than 50 cells were counted. In doubt the colony size was checked with a preparation microscope (Nikon, 40407 Düsseldorf, Germany).

3. Acceptance criteria

The gene mutation assay was considered acceptable if it meets the following criteria:

- The numbers of mutant colonies per 10^6 cells found in the solvent controls fell within the laboratory historical control data range
- The positive control substances must produce a significant increase in mutant colony frequencies
- The cloning efficiency II (absolute value) of the solvent controls must exceed 50 %.

The data of this study comply with the above mentioned.

4. Assessment criteria

A test item was classified as positive if it induced either a concentration-related increase of the mutant frequency or a reproducible and positive response at one of the test points.

A test item producing neither a concentration- related increase of the mutant frequency nor a reproducible positive response at any of the test points was considered to be non-mutagenic in this system.

A positive response was described as follows:
A test item was classified as mutagenic if it reproducibly induced a mutation frequency that was three times above the spontaneous mutation frequency at least at one of the concentrations in the experiment. The test item was classified as mutagenic if there was a reproducible concentration-related increase of the mutation frequency. Such evaluation may be considered also in the case that a threefold increase of the mutant frequency was not observed.
However, in a case by case evaluation this decision depends on the level of the corresponding solvent control data. If there was by chance a low spontaneous mutation rate within the laboratory's historical control data range, a concentration-related increase of the mutations within this range has to be discussed. The variability of the mutation rates of solvent controls within all experiments of this study was also taken into consideration.

5. Statistical analysis
A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. The number of mutant colonies obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

II. Results and discussion

No relevant cytotoxic effects and no precipitation occurred up to the maximum concentration of 1640 µg/mL with and without metabolic activation following 4 and 24 hours treatment. There was no relevant shift of pH and osmolarity of the medium even at the maximum concentration of the test item. Therefore, the maximum concentration in the main experiments was 1640 µg/mL corresponding to 10 mM of the test item.

No relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration. The mutant frequency generally remained well within the historical range of solvent controls. The induction factor exceeded the threshold of three times the mutant frequency of the corresponding solvent control at 410 and 820 µg/mL in the first culture of the first experiment without metabolic activation. This effect however, was judged as biologically irrelevant since all absolute values of the mutation frequency remained within the range of historical solvent controls. Furthermore, the increase was not reproduced in the parallel culture performed under identical experimental conditions and was not dose dependent as indicated by the lacking statistical significance.
A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequency. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in the first culture of the second experiment without metabolic activation and in the second culture of the second experiment with metabolic activation. Both trends were judged as irrelevant however, as all of the individual levels of the mutation frequency remained well within the range of historical solvent controls.
In both experiments of this study (with and without metabolic activation) the range of the solvent controls was from 6.4 up to 30.0 mutant colonies per 10^6 cells; the range of the groups treated with the test item was from 5.7 up to 33.2 mutant colonies per 10^6 cells.

EMS (150 µg/mL) and DMBA (1.1 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies.

Table 5.8-18: Mean mutant colonies per 10^6 cells in experiment I

Test groups	Concentration µg/mL	S9 mix	Culture I		Culture II	
			Mutant colonies per 10^6 cells	Induction factor	Mutant colonies per 10^6 cells	Induction factor
Control deionized water	-	-	6.4	1.0	9.6	1.0
Positive control EMS	150.0	-	100.4	15.8	68.7	7.1
Test item	51.3	-	Not continued	Not continued	Not continued	Not continued
	102.5	-	5.7	0.9	11.5	1.2
	205.0	-	13.5	2.1	13.7	1.4
	410.0	-	26.8	4.2	6.4	0.7
	820.0	-	19.8	3.1	11.0	1.1
	1640.0	-	15.2	2.4	16.4	1.7
Control DMSO	-	+	7.4	1.0	18.9	1.0
Positive control DMBA	1.1	+	752.9	102.2	640.2	33.9
Test item	51.3	+	Not continued	Not continued	Not continued	Not continued
	102.5	+	20.7	2.8	11.2	0.6
	205.0	+	17.9	2.4	15.7	0.8
	410.0	+	18.0	2.4	9.5	0.5
	820.0	+	10.5	1.4	15.9	0.8
	1640.0	+	14.1	1.9	8.1	0.4

Table 5.8-19: Mean mutant colonies per 10^6 cells in experiment II

Test groups	Concentration µg/mL	S9 mix	Culture I		Culture II	
			Mutant colonies per 10^6 cells	Induction factor	Mutant colonies per 10^6 cells	Induction factor
Control Deionized water	-	-	17.5	1.0	30.0	1.0
Positive control EMS	150.0		372.5	21.2	381.8	12.7
Test item	51.3	-	Not continued	Not continued	Not continued	Not continued
	102.5	-	15.9	0.9	21.1	0.7
	205.0	-	16.4	0.9	21.5	0.7
	410.0	-	22.3	1.3	16.9	0.6
	820.0	-	16.7	1.0	16.7	0.6
	1640.0	-	31.6	1.8	22.3	0.7
Control DMSO	-	+	24.2	1.0	82	1.0
Positive control EMS	150.0	+	773.8	32.0	596.2	72.3
Test item	51.3	+	Not continued	Not continued	Not continued	Not continued
	102.5	+	14.3	0.6	11.6	1.4
	205.0	+	31.9	1.3	11.6	1.4
	410.0	+	33.2	1.4	11.8	1.4
	820.0	+	21.8	0.9	12.4	1.5
	1640.0	+	27.4	1.1	15.0	1.8

III. Conclusions

In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.
Therefore, BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960) is considered to be non-mutagenic in this HPRT assay.

In vitro *genotoxicity (somatic cells) - Bone marrow or micronucleus*

Report:	**KIIA 5.8/10, [redacted] C.; 2011**
Title:	Micronucleus assay in the bone marrow cells of the mouse with BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960)
Report No & Document No	1421401 M-420540-01-2
Guidelines:	OECD 474 (1997); EEC Directive 440/2008/EC Method B12 (2008); EPA Health Effects Test Guideline (OPPTS 870.5395; 1998)
GLP	Yes (certified laboratory)

Executive Summary

In a pre-experiment using two males and two females per group, the following doses were used: 500, 750 and 1000mg/kg of BYI 02960-difluoroethyl-amino-furanone. A second group of two males was treated with 500 mg/kg of BYI 02960-difluoroethyl-amino-furanone. The animals received the test item dissolved in sterile water twice intraperitoneally at 24 hours interval. The animals administered at 1000 and 750 mg/kg died within two to four hours after the first administration. No animals died at 500 mg/kg in both sexes. Clinical signs such as reduction of spontaneous activity, eyelid closure and ruffled fur were rapidly observed after each administration. Based on these findings, 500 mg/kg BYI 02960 was chosen as the MTD. Due to the results of the dose range finder, it is concluded that there are no substantial differences between sexes in toxicity. Therefore, no females were used.

Each group comprised 7 male mice. The negative control group was treated with two intraperitoneal injections of sterile water, and the positive control group received only one intraperitoneal injection of cyclophosphamide at 40 mg/kg. BYI 02960-difluoroethyl-amino-furanone treated groups also received two intraperitoneal injections separated by 24 hours. The dose levels investigated in the mutagenicity experiment were: 125, 250 and 500 mg/kg.

Bone marrow from the femura from each animal was sampled 24 hours after the last intraperitoneal injection. Slides of bone marrow cells were prepared and scored for the occurrence of micronucleated polychromatic erythrocytes (micronucleated PCEs), of micronucleated normochromatic erythrocytes and PCE/total erythrocytes ratios. 2000 polychromatic erythrocytes were counted per animal.

Males treated twice with BYI 02960-difluoroethyl-amino-furanone in doses up to 500 mg/kg showed symptoms of toxicity after administration, starting at 250 mg/kg. These symptoms demonstrate relevant systemic exposure of males to BYI 02960-difluoroethyl-amino-furanone. However, all males survived until the end of the test. There was no altered ratio between polychromatic and normochromatic erythrocytes. After two intraperitoneal treatments of males with doses up to and including 500 mg/kg no indications of a clastogenic effect of BYI 02960-difluoroethyl-amino-furanone were found. Cyclophosphamide, the positive control, had a clear clastogenic effect, as is shown by the biologically relevant increase in polychromatic erythrocytes with micronuclei.

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered BYI 02960-difluoroethyl-amino-furanone in the micronucleus test on the male mouse, *i.e.* in a somatic test system *in vivo*.

I. Materials and Methods

A. Material

1. Test Material:		BYI 02960-difluoroethyl-amino-furanone
Description:		White powder
Lot/Batch:		NLL 8671-6-1
Purity:		98.5%
CAS:		1134834-71-1
Stability of test compound:		No analysis performed during the study
2. Control materials:	Negative:	None
	Solvent:	Sterile water
	Positive:	cyclophosphamide (Fisher Scientific), 40 mg/kg dissolved in sterile water (10mL/kg)
3. Test animals:		
Species:		Mouse
Strain:		NMRI
Age:		9 to 10 weeks approximately
Weight at dosing:		37 to 43 g (males only)
Source:		[redacted]
Number of animals per dose:		Range-finding test: 2 animals/sex
		Micronucleus assay: 7 males/group
Animal husbandry:		The animals were properly maintained

4. Test compound concentrations:

Range-finding test: 2 intraperitoneal injections of 500mg/kg, 750mg/kg and 1000mg/kg separated by 24 hours

Micronucleus assay: 0, 125, 250 and 500 mg/kg

B. Test performance

The experimental phase of the study was performed from July 05 to August 09, 2011 at [redacted] Germany.

1. Treatment and sampling times

Sampling of the bone marrow took place 24 hours after the last intraperitoneal injection; the positive control was sampled at 24 hours after the only one intraperitoneal injection.

2. Preparation of the animals

The animals were sacrificed using CO2 followed by bleeding. The femora were removed, the epiphyses were cut off and the marrow was flushed out with foetal calf serum, using a syringe. The cell suspension was centrifuged and the supernatant discarded. A small drop of the resuspended cell pellet

was spread on a slide. The smear was air-dried and then stained with May-Grünwald/Giemsa. Cover slips were mounted with EUKITT. Two slides were made from each bone marrow sample.

3. Details of slide preparation

Evaluation of the slides was performed using NIKON microscopes with 100x oil immersion objectives. Per animal 2000 polychromatic erythrocytes (PCE) were analyzed for micronuclei. To describe a cytotoxic effect the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and expressed in polychromatic erythrocytes per 2000 erythrocytes. The analysis was performed with coded slides.

4. Acceptance criteria

The study was considered valid as the following criteria were met:

- At least 5 animals per group can be evaluated.
- PCE to erythrocyte ratio should not be less than 20% of the negative control.
- The positive control shows a statistically significant and biological relevant increase of micronucleated PCEs compared to the vehicle.

5. Evaluation of the results

A test item is classified as mutagenic if it induces either a dose-related increase or a clear increase in the number of micronucleated polychromatic erythrocytes in a single dose group. Statistical methods (nonparametric Mann-Whitney test) were used as an aid in evaluating the results. However, the primary point of consideration is the biological relevance of the results.
A test item that fails to produce a biological relevant increase in the number of micronucleated polychromatic erythrocytes is considered to be non-mutagenic in this system.

II. Results and discussion

A. Range-finding test

The following doses were used: 500mg/kg, 750mg/kg and 1000mg/kg BYI 02960-difluoroethyl-amino-furanone. The animals administered at 1000 and 750 mg/kg died within two to four hours after the first administration. No animals died at 500 mg/kg in both sexes. Clinical signs such as reduction of spontaneous activity, eyelid closure and ruffled fur were rapidly observed after each administration. These clinical signs were usually not observed 24 hours after the second administration.

Based on these findings, 500 mg/kg BYI 02960 were chosen as MTD. Due to the results of the dose range finder it was concluded, that there were no substantial differences between sexes in toxicity. Therefore, no females were used.

B. micronucleus assay

1. Toxicity

After two intraperitoneal administrations of 250 and 500 mg/kg, BYI 02960-difluoroethyl-amino-furanone treated males showed the following compound-related symptoms: reduction of spontaneous activity, abdominal position, eyelid closure and ruffled fur. These clinical signs were observed in most animals treated at 500 mg/kg after both injections. Most of them disappeared 24 hours after injection.

Clinical signs were recorded in animals treated at 250 mg/kg after the first injection. After the second injection only one animal presented an abdominal position one hour after injection. Symptoms were recorded until sacrifice. These symptoms demonstrate relevant systemic exposure of males to BYI 02960-difluoroethyl-amino-furanone. There was no mortality. No symptoms were recorded for the control groups and the animals treated at 125 mg/kg.

2. PCE ratio

The ratio of polychromatic to normochromatic erythrocytes in males was not altered by the treatment with BYI 02960-difluoroethyl-amino-furanone.

3. Micronucleated polychromatic erythrocytes

In comparison to the corresponding vehicle control values there was no biologically relevant enhancement in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test item. The mean values of micronuclei observed after treatment with BYI 02960-difluoroethyl-amino-furanone were below the value of the vehicle control group for the low dose. A dose dependent increase occurred within the three test item dose groups resulting in micronuclei frequencies above the concurrent vehicle group in the mid and high dose groups. However, all values were within the laboratory's historical control data range and additionally, one isolated increased value of micronuclei was observed in the high dose group. Therefore, the effect is considered to be not biologically relevant.

Cyclophosphamide administered intraperitoneally once at 40 mg/kg was used as positive control which showed a statistically significant increase of induced micronucleus frequency.

Table 5.8-20: Summary of micronucleus test results

Test substance	Dose mg/kg	PCEs with micronuclei (%)	Range	PCE per 2000 erythrocytes
Vehicle	0	0.100	0 - 4	1158
BYI 02960-difluoroethyl-amino-furanone	125	0.086	0 - 4	1120
	250	0.114	1 - 3	1118
	500	0.164	2 - 7	1046
Cyclophosphamide	40	1.964	28 - 55	1039

III. Conclusions

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered BYI 02960-difluoroethyl-amino-furanone in the micronucleus test on the male mouse, *i.e.* in a somatic test system *in vivo*.

In vitro *genotoxicity (somatic cells) - DNA repair or mouse spot test*

Report:	**KIIA 5.8/11, [redacted]C.; 2011**
Title:	*In vivo* unscheduled DNA synthesis in rat hepatocytes with BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960)
Report No & Document No	1421402 M-420111-01-2
Guidelines:	OECD 486 (1997); EEC Directive 2000/32, - Method B.39 (2000), EPA Health Effects Test Guideline (OPPTS 870.5550; 1998)
GLP	Yes (certified laboratory)

Executive Summary

The test item BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960) was assessed in the *in vivo* UDS assay for its potential to induce DNA repair (UDS) in the hepatocytes of rats.
The test item was formulated in sterile water, which was used as vehicle control. The volume administered orally to Wistar rats was 10 mL/kg body weight. After a single oral treatment and a post-treatment period of 4 and 16 hours, respectively, the animals were anesthetized and sacrificed by liver perfusion. Primary hepatocyte cultures were established and exposed for 4 hours to ^{3}HTdR (methyl-^{3}H-thymidine), which is incorporated if UDS occurs.
The test item was tested at the following dose levels: 4 and 16 hours preparation intervals: 1000 and 2000 mg/kg.
The highest dose was estimated in a pre-experiment to be the maximum applicable dose, at which clinical signs of toxicity occurred without affecting the survival rates.
For each experimental group including the controls, hepatocytes from at least three treated animals were assessed for the occurrence of UDS.
The viability of the hepatocytes was not substantially affected by the *in vivo* treatment with the test item.
Ruffled fur and reduction of spontaneous activity were also indicative of systemic toxicity.
None of the tested dose levels revealed UDS induction in the hepatocytes of the treated animals as compared to the corresponding vehicle controls.
Appropriate reference mutagens (DMH, 80 mg/kg and 2-AAF, 100 mg/kg) were used as positive controls. Treatment with positive control substances revealed distinct increases in the number of nuclear and net grain counts.

In conclusion, under an oral administration up to 2000 mg/kg, the test item did not induce DNA-damage leading to increased repair synthesis in the hepatocytes of the treated rats. Therefore, BYI 02960-difluoroethyl-amino-furanone is considered to be non-genotoxic in this *in vivo* UDS test system.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960-difluoroethyl-amino-furanone
Description:	White powder
Lot/Batch:	NLL 8671-6-1
Purity:	98.5%
CAS:	1134834-71-1
Stability of test compound:	No analysis performed during the study
2. Control materials: Negative:	None
Solvent:	Sterile water
Positive:	N,N'-dimethylhydrazinedihydrochloride (DMH, Sigma-Aldrich) at 80 mg/kg in 0.9% NaCl solution for 4 hours preparation interval;2-acetylaminofluorene (2-AAF, Sigma-Aldrich) at 100 mg/kg in DMSO/PEG 400 (1 + 9).
3. Test animals:	
Species:	Rat
Strain:	Wistar
Age:	6 weeks approximately
Weight at dosing:	181.7 ± 13.2 g (males only in the main experiment)
Source:	[redacted] Netherlands
Number of animals per dose:	Range-finding test: 2 animals/sex/group Main experiment: 4 males/dose/time interval
Animal husbandry:	The animals were properly maintained
4. Test compound concentrations:	
Range-finding test:	2000 mg/kg by gavage
Main experiment:	1000 and 2000 mg/kg by gavage with 4 and 16 hours treatment periods

B. Test performance

The experimental phase of the study was performed from July 05 to August 31, 2011 at [redacted] Germany.

1. Pre-experiment on Toxicity

A preliminary study on acute toxicity was performed with two animals per group and sex. The animals were treated orally by gavage at 2000 mg/kg and examined for acute toxic symptoms at intervals of 1 hour, 2 - 4 hours, 6 hours and 24 hours after administration of the test item.

Reduced spontaneous activity and ruffled fur were observed in all animals between 1 hour after administration and 24 hours. Between 2 hours and 6 hours eyelid closure was also observed in both sexes. On the basis of these data, 2000 mg/kg was considered the maximum tolerated dose. Since no

gender specific differences on acute toxic symptoms were observed, the main study was performed using males only.

2. Animal treatment in the main experiment

Four male Wistar rats were assigned in each test group and each post-treatment period. There were one negative control, one treated group with the test item at 1000 mg/kg, one treated group with the test item at 2000 mg/kg and one positive control group (DMH was used as positive control for the 4 hours preparation interval and 2-AAF was used as positive control for the 16 hours preparation interval). The administration volume was 10 mL/kg bw.

Before the beginning of the treatment the animals were weighed and the individual volume to be administered was adjusted to the body weight of the animals. The animals received the test item once and examined for acute toxicity at intervals of 1, 2 and 4 hours for the 4 hours treatment and 1 and 16 hours for the 16 hours treatment.

3. Isolation of the primary hepatocytes

After anaesthetizing the rats with 46% Ketamine, 23% Xylazin and 31% Midazolan (approximately 2 mL/kg bw), the liver was perfused through the vena portae with Hank's balanced salt solution supplemented with collagenase (0.05%) adjusted to pH 7.4 and maintained at 37 °C.

The isolated hepatocytes were washed twice with HBSS. The crude cell suspension was filtered through a stainless steel mesh to yield a single cell suspension. The quality of the performed perfusion was determined by trypan blue dye exclusion method for cell viability. In addition, the number of cells was determined.

4. Culture conditions

The washed hepatocytes were centrifuged and transferred into Williams medium supplemented with Hepes (2.38 mg/mL), L-Glutamine (0.29 mg/mL), Insulin (0.50 µg/mL), Penicillin (100 units/mL), Streptomycin (0.10 mg/kg) and fetal calf serum (100 µg/mL). This complete medium was adjusted to pH 7.6.

At least three cultures were established from each animal. Aliquots of 2.5 mL with freshly isolated hepatocytes in complete culture medium (2.0×10^5 viable cells/mL) were added to 35 mm six-well dishes containing one 25 mm round plastic coverslip per well coated with gelatin.

After an attachment period of approximately 1.5 hours in a 95% air/5% CO_2 humidified incubator at 37 °C the culture medium was discarded. Then the cell layer was rinsed once with PBS to remove non-adherent cells. Subsequently, ^{3}HTdR (5 µCi/mL, specific activity 20 Ci/mmol) in 2.0 mL culture medium supplemented with 1% fetal calf serum (FCS) was added to the cultures. After a labeling time of 4 hours the cells were washed twice with culture medium supplemented with 1% FCS and 0.25 mM unlabeled thymidine. Cultures were incubated overnight using the same medium. To prepare for autoradiography the medium was replaced by a hypotonic solution of 1% sodium citrate for 10 minutes to swell the nuclei for better grain detection. The cells on the coverslips were then fixed by three changes of methanol:acid acid (3+1 v/v) for 20 minutes each, rinsed with 96% (v/v) ethanol and air-dried.

5. Autoradiography processing

The cover slips were mounted the side carrying the cells up on glass slides and coated with Kodak NTB photographic emulsion in the dark. The coated slides were stored in light-proof boxes in the presence of a drying agent for 14 days (except the reserved slides for 7 days) at 4 °C. The photographic emulsion was then developed with Ilford Phenisol at room temperature, fixed in Rapid Fixer and stained with hematoxylin/eosin.

6. Quantification of UDS

Evaluation was performed microscopically on coded slides using Nikon microscopes with oil immersion objectives. The cells for scoring were randomly selected according to a fixed scheme. The number of silver grains in the nuclear area was counted automatically using the sorcerer UDS device. In addition, the number of grains of the most heavily labeled nuclear-sized cytoplasm area adjacent to the nucleus was counted. At least two slides per animal and 50 cells per slide were evaluated. Heavily radio-labeled cells undergoing replicative DNA synthesis were excluded from counting. At least three animals per group were evaluated as described above.
The nuclear and cytoplasmic grain counts, the net grain counts (nuclear minus cytoplasmic grains) as well as the mean and percentage of cells in repair (cells with a net grain count larger than 5) were reported separately. The mean counts with standard deviation were used to describe the distribution of ^{3}HTdR incorporation in the nucleus, the cytoplasm and for the net grains, respectively.

7. Evaluation of results

Nuclear and net grain counts are estimated together. Increased net grains should be based on enhanced nuclear grains counts rather than on decreased cytoplasmic grain counts.

A test item is classified as positive if the mean number of net grains is higher than five per nucleus at one of the test points.

A group average between 0 and 5 net grains is considered as a marginal response. A dose-related increase in nuclear and net grains and/or a substantial shift of the percentage distribution of the nuclear grain counts to higher values provide additional information to confirm a positive response with less than 5 net grains.

Statistical significance may give further evidence for a positive evaluation. Statistical significance can be evaluated by means of the non-parametric Mann-Whitney test.

A test item producing net grains not greater than 0 at anyone of the test points is considered non-effective in this system.

A statistical evaluation of the results was not necessary as the number of net grain counts of the groups treated with the test item were in the range of the corresponding controls.

II. Results and discussion

A. Toxicity evaluation

In the main experiment with 4 hours treatment period, the four males which received the test item at 1000 mg/kg showed reduced spontaneous activity and ruffled fur at 1, 2 and 4 hours. The same clinic signs were observed in the animals treated at 2000 mg/kg, but, reduced activity was observed in 2/4 rats at 1 hour, 3/4 rats at 2 hours and all animals at 4 hours. Ruffled fur was observed in 1/4 rats at 1 hour, 3/4 rats at 2 hours and all animals at 4 hours.
In the main experiment with 16 hours treatment period, all animals treated at 1000 mg/kg showed reduced spontaneous activity and ruffled fur at 1 and 16 hours and all animals treated at 2000 mg/kg showed reduced spontaneous activity and ruffled fur at 4 and 16 hours except for ruffled fur at 1 hour when only 2 rats represented this sign.

B. UDS Quantification

The viability of the hepatocytes was not substantially affected by the *in vivo* treatment with the test item at any of the treatment periods or dose groups. The inter-individual variations obtained for the numbers and the viabilities of the isolated hepatocytes were in the range of the historical laboratory control.
No dose level of the test item revealed UDS induction in the hepatocytes of the treated animals as compared to the current vehicle controls. Neither the nuclear grain counts nor the resulting net grain counts were distinctly increased after *in vivo* treatment of the animals with the test item at 4 hours or 16 hours, respectively. Net grain counts obtained after treatment with the test item remained consistently negative. In addition, no substantial shift to higher values of percentage of cells in repair was reported.
Appropriate reference mutagens (DMH at 80 mg/kg and 2-AAF at 100 mg/kg) were used as positive controls. *In vivo* treatment with DMH or 2-AAF revealed distinct increases in the number of nuclear and net grain counts.

Table 5.8-21: Summary of the 4 hours treatment experiment

Test group	Animal number	Mean Net grain count (mean ± SD)	Mean nuclear grains of cells in Repair (mean ± SD)	% Cells in repair
Sterile Water	1	- 6.55 ± 6.12	7.33 ± 1.53	3
	2	- 7.90 ± 5.69	9.00 ± 0.00	2
	3	- 13.08 ± 8.54	0.00 ± 0.00	0
	4	- 8.79 ± 7.92	10.00 ± 7.07	2
Test item 1000 mg/kg	5	- 8.96 ± 9.86	6.80 ± 2.49	5
	6	- 7.61 ± 5.39	0.00 ± 0.00	0
	7	- 7.47 ± 6.65	6.50 ± 0.71	2
	8	- 11.06 ± 7.72	11.00 ± 0.00	1
Test item 2000 mg/kg	9	- 9.42 ± 6.81	0.00 ± 0.00	0
	10	- 10.21 ± 8.07	8.33 ± 3.06	3
	11	- 16.47 ± 13.71	9.25 ± 2.50	4
	12	- 10.05 ± 8.08	6.33 ± 0.58	3
DMH 80 mg/kg	13	32.74 ± 15.84	33.05 ± 15.61	99
	14	49.41 ± 18.41	49.41 ± 18.41	100
	15	27.67 ± 12.02	27.67 ± 12.02	100
	16	37.16 ± 23.21	40.45 ± 21.15	92

Table 5.8-22: Summary of the 16 hours treatment experiment

Test group	Animal number	Mean Net grain count (mean ± SD)	Mean nuclear grains of cells in Repair (mean ± SD)	% Cells in repair
Sterile Water	17	- 15.22 ± 7.62	7.00 ± 0.00	1
	18	- 11.09 ± 7.50	0.00 ± 0.00	0
	19	-12.99 ± 9.40	5.00 ± □0.00	2
	20	- 8.87 ± 6.30	13.00 ± 0.00	1
Test item 1000 mg/kg	21	- 9.67 ± 10.76	8.86 ± 2.34	7
	22	- 9.53 ± 8.27	9.00 ± 0.00	1
	23	- 10.23 ± 8.68	11.00 ± 0.00	1
	24	-9.95 ± 8.97	6.50 ± 1.00	4
Test item 2000 mg/kg	25	- 14.65 ± 9.28	0.00 ± 0.00	0
	26	-17.00 ± 10.20	0.00 ± 0.00	0
	27	- 14.28 ± 8.39	8.00 ± 0.00	1
	28	- 10.27 ± 6.52	5.00 ± 0.00	1
2-AAF 100 mg/kg	29	16.83 ± 10.08	18.19 ± 8.92	93
	30	12.97 ± 8.16	14.85 ± 7.11	86
	31	12.16 ± 7.88	14.21 ± 6.26	86
	32	14.05 ± 8.83	16.76 ± 6.76	83

III. Conclusions

In conclusion, oral administration up to the maximal tolerated dose of 2000 mg/kg, BYI 02960-difluoroethyl-amino-furanone did not induce DNA-damage leading to increased repair synthesis in the hepatocytes of the treated rats.

Acute oral toxicity

Report:	**KIIA 5.8/12, [redacted] N.; 2011**
Title:	BYI 02960-difluoroethyl-amino-furanone, Acute toxicity in the rats, "Acute toxic class method".
Report No & Document No	37503 TAR M-409674-01-2
Guidelines:	OECD 423 (2001); EEC Directive 440/2008 - Method B.1.tris (2008)
GLP	Yes (certified laboratory)

Executive Summary

In an acute oral toxicity study using a stepwise procedure, three groups of three fasted, young adult female Sprague Dawley rats were given successively a single oral dose of BYI 02960-difluoroethyl-amino-furanone (batch NLL 8671-6-1; 98.5% purity) in purified water of 300, 2 000 and then 2000 mg/kg bw and were observed for 14 days.
Each animal was observed at least once a day for mortality and clinical signs for 15 days. Body weight was recorded on day 1 and then on days 8 and 15. On completion of the observation period, the animals were sacrificed and then submitted for a macroscopic *post-mortem* examination. For all animals, macroscopic lesions were preserved and no microscopic examination was performed.

No unscheduled deaths occurred during the study.

At 2000 mg/kg, hypoactivity, piloerection and/or chromorhynorrhea were noted in first and/or confirmatory assay females within 4 hours after treatment. No clinical signs persisted on day 2. Low body weight gain was observed in 2/3 females (first assay) all over the study period and in 1/3 females (confirmatory assay) between days 1 and 8.

At 300 mg/kg, no clinical signs were noted in any animals. Body weight was unaffected by the treatment.

The test item administration did not induce any macroscopic findings.

Under the experimental conditions of this study, the oral LD_{50} of BYI-02960-difluoroethyl-amino-furanone, was higher than 2000 mg/kg in rats. Therefore, the test item is not classified as toxic by oral route according to the criteria of CLP Regulation.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960(difluoroethyl-amino-furanone
Description:	White powder
Lot/Batch:	NLL 8671-6-1
Purity:	98.5%
CAS:	1134834-71-1
Stability of test compound:	No analysis performed during the study
2. Vehicle and /or positive control:	Drinking water treated by reverse osmosis
3. Test animals:	
Species:	Rat
Strain:	Rj Han: SD (IOPS Han)
Age:	8 weeks approximately
Weight at dosing:	202 to 219 g
Source:	[redacted] France
Acclimation period:	7 days
Diet:	[redacted]R/M-H pelleted diet, batch No. 7134735 and 9945009 ([redacted] Germany), *ad libitum*
Water:	Tap water filtered with a 0.22 µm filter, *ad libitum*
Housing:	Animals were group caged conventionally in polycarbonate cages on autoclaved sawdust
Environmental conditions:	Temperature: 22 ± 2 °C
	Humidity: 50 ± 20%
	Air changes: Approximately 12 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles.

B. Study Design and methods

1. In life dates

February 23 to March 24, 2011 performed at [redacted] France).

2. Animal assignment and treatment

The substance was tested using a stepwise procedure, each step using three female rats. The animals were assigned to their groups by randomization based on a computerized random procedure. Following an overnight fast (18 hours), the first group received a single dose of 300 mg/kg of BYI 02960-difluoroethyl-amino-furanone (98.5% purity) by gavage. The test substance was administered in drinking water treated by reverse osmosis at a volume of 10 mL/kg bw. After the first assay, as no deaths occurred, another assay was carried out on three animals at the next higher dose-level (2000 mg/kg). After the second assay, as no deaths occurred, the results were confirmed in three other females at the dose-level of 2000 mg/kg. Clinical signs and mortality rates were determined several times on the

day of administration and subsequently at least once daily for an observation period of at least 14 days. Body weights were recorded on days 1, 8 and 15. On day 15, surviving animals were sacrificed and all animals were necropsied and examined for gross pathological changes.

3. Statistics

The data did not warrant statistical analysis.

II. Results and discussion

A. Mortality

No unscheduled deaths occurred during the study.

B. Clinical observations

At 2000 mg/kg, hypoactivity was noted in one female (first assay) between 1 hour and 4 hours after treatment. In the confirmatory assay, piloerection was observed in three females 4 hours after treatment, associated with chromorhynorrhea in one female. No clinical signs persisted on day 2 in these animals.

At 300 mg/kg, no clinical signs were noted in any animals.

C. Body weight

At 2000 mg/kg, lower mean body weight gain was observed in groups 2 females all over the study period (-28%) and in group 3 females between days 1 and 8 (-10%), when compared to CIT historical control data. These variations were due to lower body weight gain in 2/3 females (first assay) and 1/3 females (confirmatory assay).
Body weight of the animals treated at 300 mg/kg was unaffected by test item treatment, when compared to CIT historical control data.

D. Necropsy

The few macroscopic findings noted at the end of the treatment period were of those commonly recorded in the Sprague-Dawley rat and none were considered to be related to the test item administration.

III. Conclusions

Under the experimental conditions of this study, the oral LD_{50} of BYI-02960-difluoroethyl-amino-furanone was higher than 2000 mg/kg in rats. Therefore, the test item is not classified as toxic by oral route according to the criteria of CLP Regulation. The results meet the criteria for EPA toxicity category III.

Oral 14-day toxicity in the rat

Report:	**KIIA 5.8/13; [redacted] R.; 2012**
Title:	BYI 02960-difluoroethyl aminofuranone: A 14-day dose range finding toxicity/palatability study in rats
Report No & Document No	11/116-100PE; M-426158-01-2
Guidelines:	Not applicable (only preliminary and explorative study design)
GLP	Study not performed under GLP, but laboratory GLP-certified

Executive summary

BYI 02960-difluoroethyl aminofuranone, a metabolite of BYI 02960 (batch number NLL 8671-12-1; a white powder, 98.9 % w/w purity) was administered continuously via the diet to groups of Wistar rats (5/sex/group) for at least 14 days at concentrations of 1280, 3200, 8000 and 20000 ppm (equating to approximately 135, 339, 736 and 1226 mg/kg body weight/day in males and 135, 335, 741 and 2254 mg/kg body weight/day in females). A similarly constituted group received untreated diet and served as a control. Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight was recorded on days -7, 0, 1, 3, 6, 7, 10, 13 and 14. Full feeders were weighed on days -7, 0, 1, 3, 6, 7 and 10 and empty feeders were weighed on days 0, 1, 3, 6, 7, 10 and 13. Hematology and clinical chemistry parameters were determined at the end of the study. All animals were necropsied, selected organs weighed and a range of tissues were retained in fixative. No histopathological examination was performed.

There was no unscheduled mortality during the study. There were no clinical signs during the 14-day administration in either the treated, or control animals.
Adverse effects considered related to BYI 02960-difluoroethyl aminofuranone administration under the conditions of this study were noted on the animal body weight and/or body weight gain values in the 3200, 8000 and/or 20000 ppm male and/or female animals, with an apparent dose response.
At 3200 ppm, statistically lower body weight gain was noted in males between Days 0 and 1 (1.4 g *vs.* 7.4 g) as well as when calculated for the whole duration of the study (Days 0 to 14, 78 g *vs.* 94.6 g, $p < 0.05$, 37.04% *vs.* 45.67 %). In the Low-mid dose 3200 ppm Group 3 females, statistically lower body weights up to -11.93% were recorded approximately after 1 week of treatment on Day 6 and towards the end of the treatment on Days 10, 13 and 14.

At 8000 ppm, statistically lower mean body weights up to -16.48% were noted in the Group 4 males after Day 3 of the treatment, and up to -15.88% in the females, after Day 6, until its completion on Day 14.
In the 20000 ppm Group 5 animals, body weight loss was noted in both males and females between Days 0 and 14.

The mean daily food consumption evaluated based on the cage-based weekly food consumption of male and female rats was lower than control in the 8000 and 20000 ppm Groups 4 and 5, up to - 59% lower in the males and up to - 25% in the female at 20000 ppm, effects considered possibly associated with test item administration. However, spillage was noted in the High dose Group 5 females and the dose response was not clear.

Statistically lower than control reticulocyte mean values were noted in the 20000 ppm males and 8000 and 20000 ppm females and were regarded as possibly associated with BYI 02960-difluoroethyl aminofuranone administration.

Lower than control glucose blood levels were noted in both males and females in all the dose groups, attaining statistical significance at above and including 3200 ppm in the males, with mean values up to 49% lower than control, and at all the dose levels tested in the females, with mean values of up to - 52%, p <0.01. Cholesterol mean values increased in the 3200 ppm group and above to up to 65% in the males, and up to 49% in the females. Albumin and A/G ratio were slightly higher than control, attaining statistical significance at 8000 and/or 20000 ppm in the males and in both males and females, respectively.

In addition, higher urea was noted in the 20000 ppm males, and in the 20000, and 8000 ppm females. Potentially test item-related macroscopic changes were observed at necropsy in the High dose group fed diet with 20000 ppm BYI 02960-difluoroethyl aminofuranone. These consisted of small prostate and seminal vesicles in 5/5 males and small spleen, in 2/5 males, were associated with the potential test item related changes in the terminal body weights and correlated with the organ weight changes; however, a definitive attribution cannot be made without histopathological evaluation.

In conclusion, based on the effects noted in the current 14-day preliminary study and in correlation with the previous data available at the Sponsor, the dose levels selected were 200, 800 and 3000 ppm (mg/kg diet). These dose levels were considered to be suitable for an upcoming 28-day dietary study with BYI 02960-difluoroethyl aminofuranone.

I. Materials and Methods

A. Material

1. Test Material: BCS-CC98193, BYI 02960-difluoroethyl aminofuranone

Description:	White powder
Lot/Batch:	NLL 8671-12-1
Purity:	98.9%
CAS:	1133834-71-1
Stability of test compound:	Stable from 500 to 20000 ppm for at least 7 weeks in the diet storage room (approximately 15 - 21 °C) and at least 14 days in the animal room (approximately 22 ± 3 °C)

2. Vehicle and /or positive control: None

3. Test animals:

Species:	Rat
Strain:	Crl:WI rats
Age:	7 weeks
Weight at dosing:	196 to 227 g for the males; 148 to 174 g for the females

Source: [redacted] Germany
Acclimation period: 2 weeks
Diet: [redacted]® SM R/M-Z+H "Autoclaved Complete Feed for rats and mice - Breeding and maintenance" (by [redacted] Germany), *ad libitum*
Water: Tap water, *ad libitum*
Housing: Animals were group-housed (5 animals/sex/cage)

Environmental conditions:
Temperature: 20.1 - 24.1 °C
Humidity: 31 - 68%
Air changes: 15 to 20 changes per hour
Photoperiod: Alternating 12-hour light and dark cycles (6 am - 6 pm)

B. Study Design

1. In life dates
15 to 29 September 2011 at [redacted] - Hungary.

2. Animal assignment and treatment
There were 5 rats per sex per dose group. Animals were assigned to dose groups using a randomization by weight. BYI 02960-difluoroethyl aminofuranone was administered in the diet for 14 days to Wistar rats at the following doses: 0, 1280, 3200, 8000 and 20000 ppm (equating to approximately 135, 339, 736 and 1226 mg/kg/day in males and 135, 335, 741 and 2254 mg/kg/day in females). A negative control group received plain diet.

Table 5.8.2-3: Study design

Test Group	Diet Concentration (ppm)	Animals assigned	
		Male	Female
1/Control	0	5	5
2/Low dose	1280	5	5
3/Low mid dose	3200	5	5
4/High mid dose	8000	5	5
5/High dose	20000	5	5

3. Diet preparation and analysis
BYI 02960-difluoroethyl aminofuranone was incorporated into [redacted]® SM R/M-Z+H "Autoclavable Complete Feed for Rats and Mice - Breeding and Maintenance" by [redacted] Germany and mixed for up to approximately 14 minutes (approximately 6 minutes for premix preparation, and 4-8 minutes for preparation of the complete diets), in a room where the temperature and humidity were controlled. Following mixing, pellets were prepared by simple compression; no binding agents, steam, external heat, any other process or substance were used that

might affect BYI 02960-difluoroethyl aminofuranone or the quality of the diets. Similar diet preparation procedures were used to generate control diet (0 mg BYI 02960-difluoroethyl aminofuranone/kg diet). The prepared diets were stored at room temperature under dry conditions, in sewed bags pending and during transport to [redacted] Hungary Ltd. At [redacted] Hungary Ltd., the prepared diets were stored in areas designated for diet storage at room temperature (approximately 15 - 21 °C) under dry conditions, pending transfer to animal room at approximately 22 ± 3 °C for animal feeding.

Analyses of the diets for homogeneity and/or concentration of BYI 02960-difluoroethyl aminofuranone were performed based on an analytical method validated at [redacted] Hungary Ltd. (SL116-316AN). Concentration and homogeneity assessment were performed at the diet arrival; additional concentration measurements were conducted at the end of the study, from the remaining diet collected from the animal room on Day 13. At receipt of the diets five samples were taken from different areas (top, middle and bottom) of the diet containers. On Day 13 five samples were collected from the remaining diet in the animal room, from different areas (top, middle and bottom) of the hoppers. One sample was taken from the control. At receipt of the diets the samples were homogenized and three replicate extractions were carried out from each sample in order to test the homogeneity. On Day 13 only the concentration of the diet was verified. The samples were mixed and five replicate extractions were carried out.

Diet samples were stored at room temperature, dry, pending analysis on the same day. No test item was detected in the Control samples. The test item was homogenously distributed in the diet. The concentration of the test item in the diet samples varied between 91% and 102% of the nominal values, within the acceptable range of 100 ± 10% and thus these results were considered suitable for the study purposes.

4. Statistics

The heterogeneity of variance between groups was checked by Bartlett's homogeneity of variance test. Where no significant heterogeneity was detected, a one-way analysis of variance was carried out. If the obtained result was positive, Duncan's Multiple Range test was used to assess the significance of inter-group differences. Where significant heterogeneity was found, the normal distribution of data was evaluated by Kolmogorov-Smirnov test.

In case of abnormal distribution, the non-parametric method of Kruskal-Wallis One-Way analysis of variance was applied. If the result was positive, the inter-group comparisons were performed using Mann-Whitney U-test. The mean and standard deviations values, the frequency of clinical observations and macroscopic findings were calculated as applicable.

B. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily. All animals were observed for clinical signs at least once daily at approximately the same time, with minor variations as practical. Detailed physical examinations were performed on all animals outside the home cage at randomization (Day -7), on the first day of treatment and at least weekly during the treatment period. The nature, onset,

severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight

Body weights were recorded with a precision of 1 g at randomization (Day - 7), on the first day of treatment (Day 0, prior to start of treatment), then on Days 1, 3, 6, 7, 10, and 13 and 14 (fasted, to allow calculation of the relative organ weights).

3. Food consumption and compound intake

Food consumption were measured pre-treatment from Day - 7 to Day 0. The remaining, non-consumed food given as of Day 0 was weighed with a precision of 1g on Days 1, 3, 6, 7, 10, and 13 during the treatment period.

4. Clinical pathology

Blood sampling

Blood samples for clinical pathology evaluation were collected from all animals immediately prior to the scheduled necropsy on Day 14, after an overnight period of food deprivation of animals, by heart puncture under pentobarbital anaesthesia.

Three samples were taken from each animal, one for haematology (approximately 1.2 mL blood into K_3-EDTA tubes, 1.6 mg/mL blood), one for determination of blood clotting times (approximately 1.2 mL blood for APTT and PT measurements, into sodium citrate tubes) and the third one to obtain serum samples (up to 2.4 mL blood as practical, into tubes with no anticoagulant) for clinical chemistry.

Hematology

Red blood cell count, haemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cell volume, reticulocyte count, white blood cell count and differential count evaluation and platelet count and mean platelet volume were assayed using an Advia 120.

Blood smears were prepared for all animals but their examination was not considered required under the conditions of this preliminary study.

Prothrombin time and Activated partial thromboplastin time were assayed on an AMAX Destiny Plus Coagulometer.

Clinical chemistry

Total bilirubin, glucose, urea, creatinine, total protein, albumin, total cholesterol, triglycerides, phosphorus, sodium, potassium, calcium and chloride concentrations, and γ glutamyltransferase, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed using an VITROS equipment. Bile acids were evaluated on a Lory 2000.

5. Sacrifice and pathology

Necropsy and macroscopic examination were performed on all animals, which survived to the scheduled termination on Day 14, after the blood collection for clinical pathology evaluation. The animals were euthanized by exsanguination under pentobarbital anaesthesia.

After exsanguination the external appearance were examined, cranium, thoracic and abdominal cavities were opened and the appearance of the tissues and organs was observed macroscopically. Any abnormalities were recorded with details of the location, color, shape and size, as appropriate.

The following organs were weighed: Adrenal glands, brain, epipidymides, heart, kidney, liver, prostate, ovaries, seminal vesicles with coagulating glands, spleen, testes, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix). Paired organs were weighed together.
All the organs weighed as above and any organs with macroscopic abnormalities were retained in fixative as applicable (testes and epididymides in Bouin's solution, all other organs in 10% buffered formalin solution). No histopathology evaluation was performed during the study in agreement with the Sponsor, in consultation with the Study Director.

II. Results and discussion

A. Observations

1. Clinical signs of toxicity
There were no clinical signs observed during the study.

2 .Mortality
There was no unscheduled mortality during the study.

B. Body weight and body weight gain

At 20000 ppm, body weight loss was noted in both males and females between Days 0 and 14, with lower values of up to -104.02% in the males (-3.8 g *vs.* 94.6 g, - 1.88% *vs.* 45.67%) and up to -122.52% in the females (-10 g *vs.* 44.4 g, - 6.48% *vs.* 27.10%).

At 8000 ppm, statistically lower mean body weights up to -16.48% were noted in the males after Day 3 of the treatment and up to -15.88% in the females, after Day 6, until its completion on Day 14. The body weight gains between Days 0 and 14 were up to -54% lower than control in the males (43.4 g *vs.* 94.6 g, 20.76% *vs.* 45.67%) and up to -64% in the females (16 g *vs.* 44.4 g, 9.93% *vs.* 27.10%).

At 3200 ppm, statistically lower body weight gain was noted in males between Days 0 and 1 (1.4 g *vs.* 7.4 g) as well as when calculated for the whole duration of the study (Days 0 to 14, 78 g *vs.* 94.6 g, 37.04% *vs.* 45.67 %). In females, statistically lower body weights up to -11.93% were recorded approximately after 1 week of treatment on Day 6 and towards the end of the treatment on Days 10, 13 and 14. The body weight gain was also lower than control between Days 3 and 6 (4.2 g *vs.* 13.4 g) as well as when calculated for the whole duration of the study (Days 0 to 14, 26.2 g *vs.* 44.4 g, 16.76% *vs.* 27.10 %).

There were no body weight or body weight gain changes considered related to test item administration, or to reflect an adverse effect, at 1280 ppm BYI 02960-difluoroethyl aminofuranone.

Table 5.8-24: Summary of body weight and body weight gain data (g)

Dose levels of Test item (ppm)	0	1280	3200	8000	20000
Male					
Initial BW (Day 0) (%C)	207.6	211.0(102%)	210.6(101%)	209.0(101%)	208.0(100%)
BW (Day 1) (%C)	215.0	218.8 (102%)	212.0 (99%)	202.2 (94%)	198.0* (92%)
BW (Day 7) (%C)	265.0	266.0 (100%)	255.6 (96%)	235.2** (89%)	196.2** (74%)
Final BW (Day 14) (%C)	302.2	301.2 (100%)	288.6 (95%)	252.4** (84%)	204.2** (68%)
BWG (Day 0-1) (%C)	7.4	7.8 (105%)	1.4* (19%)	-6.8** (-192%)	- 10.0** (-235%)
BWG (Day 1-3) (%C)	17.6	15.4 (87%)	16.2 (92%)	6.8** (39%)	-9.4** (-53%)
BWG (Day 7-10) (%C)	30.2	26.2 (87%)	25.4 (84%)	16.0** (53%)	11.2** (37%)
Overall BWG (Days 0-14) (%C)	94.6	90.2 (95%)	78.0 (82%)	43.4** (46%)	-3.8** (-104%)
Female					
Initial BW (Day 0)(%C)	163.4	160.2 (98%)	156.8 (96%)	158.8 (97%)	155.4 (95%)
BW (Day 1) (%C)	163.0	164.2 (101%)	157.0 (96%)	153.8 (94%)	143.8** (88%)
BW (Day 7) (%C)	187.8	186.6 (99%)	174.0 (93%)	168.6* (90%)	147.4** (78%)
Final BW (Day 14) (%C)	207.8	200.4 (96%)	183.0** (88%)	174.8** (84%)	145.4** (70%)
BWG (Day 0-1) (%C)	0.4	4.0 (900%)	0.2 (50%)	- 5.0 (-1350%)	11.6** (3000%)
BWG (Day 1-3) (%C)	10.0	8.2 (82%)	8.0 (80%)	6.4 (64%)	- 2.6** (-126%)
BWG (Day 7-10) (%C)	18.2	10.6* (58%)	11.2 (62%)	13.4 (74%)	3.6** (20%)
Overall BWG (Days 0-14) (%C)	44.4	40.2 (90%)	26.2** (59%)	16.0** (36%)	- 10.0** (-123%)

* : p <0.05 ** : p <0.01

C. Food consumption and compound intake

The mean daily food consumption evaluated based on the cage-based weekly food consumption of male and female rats was lower than control in the 8000 and 20000 ppm Groups 4 and 5, up to - 59% lower in the males and up to - 25% in the female at 20000 ppm, effects considered possibly associated with test item administration. However, spillage was noted in the High dose Group 5 females, making assessment difficult.

Possible test-item related adverse effects were noted on the food conversion efficiency FCE values (g/g, calculated as weekly body weight gain g/weekly food consumption g) at 3200, 8000 and 20000 ppm, up to - 16.45%, - 30.26% and - 85.09% in the Groups 3, 4 and 5 males and - 18.84%, - 22.11% and - 85.69% in the Groups 3, 4 and 5 females, respectively.

The mean intake over the 14 days was 135, 339, 736 and 1226 mg/kg bw/day for the males and 135, 335, 741 and 2254 mg/kg bw/day for the females from Groups 2, 3, 4 and 5, respectively.

Table 5.8-25: Summary of food consumption (g)

Dose levels of Test item (ppm)	0	1280	3200	8000	20000
Male					
FC (Days 0-7) (%C)	25.4	26.6 (105%)	26.0 (102%)	20.0 (79%)	10.5 (41%)
FC (Days 7-13) (%C)	28.8	29.5 (102%)	28.4 (99%)	24.5 (85%)	15.9 (55%)
Female					
FC (Days 0-7) (%C)	19.1	18.8 (98%)	17.7 (93%)	15.0 (79%)	18.5 (97%)
FC (Days 7-13) (%C)	22.7	20.2 (89%)	19.4 (85%)	17.7 (78%)	17.1(75%)

D. Clinical pathology

1. Hematology

Statistically lower than control reticulocyte mean values were noted in the 20000 ppm males (3.04% *vs.* 5%) and 8000 and 20000 ppm females (3.16% and 2.28%, *vs.* 3.94% in the control group) and were regarded as possibly associated with BYI 02960-difluoroethyl aminofuranone administration, although the values in the test item treated groups remained only slightly lower than the physiological ranges. The white blood cell count was statistically lower than control at all the dose levels tested in the male animals and lower than concurrent historical control mean values; however, the mean values were comparable with the values recorded in the females, including control, there were no similar statistical differences in the females and thus a test item related effect was regarded as equivocal.

Other parameters showed on occasion statistically significant variations, including slightly higher red blood cell count RBC or haemoglobin HGB in the 8000 ppm and 20000 ppm males but not in the females, or lower PLT in the 3200 ppm males only. In the absence of a clear dose or gender response and/or as the values were generally comparable with the physiological ranges, these variations were not considered toxicologically significant or related to treatment.

2. Clinical chemistry

Lower than control glucose blood levels were noted in both males and females in all the dose groups, attaining statistical significance at above and including 3200 ppm in the males, with mean values up to -49% lower than control, and at all the dose levels tested in the females, with mean values of up to - 52%.

Cholesterol mean values increased in the 3200 ppm group and above to up to 65% in the males, attaining statistical significance at 8000 ppm (38%) and 20000 ppm (65%), and up to 49% in the females, attaining statistical significance in the High dose females only (49% higher than control).

Albumin and A/G ratio were slightly higher than control, attaining statistical significance at 8000 and/or 20000 ppm in the males and in both males and females, respectively.

These effects were considered to reflect a BYI 02960-difluoroethyl aminofuranone-related adverse effect at and above 3200 ppm, under the conditions of this study.

In addition, higher urea was noted in the 20000 ppm males (32%), with 20% and 15% higher values in the 20000, and 8000 ppm females, respectively, without attaining statistical significance. As the dose response was not clear, although a test item effect at 20000 ppm cannot be excluded, it cannot be ascertained in the conditions of this study. Similar changes that were considered equivocal in correlation with BYI 02960-difluoroethyl aminofuranone administration in diet mostly at 8000 and 20000 ppm were noted in the ALT, T-BIL and bile acids mean values, with statistically significant variations observed on occasion, although without a clear dose or gender response.

Other clinical chemistry parameters showed on occasion statistically significant variations, however, there was no dose or gender response or the values were within the physiological ranges. For this reason, these variations were not considered toxicologically significant or related to treatment.

Table 5.8-26: Summary of clinical chemistry changes

Dose levels of Test item (ppm)	0	1280	3200	8000	20000
Male					
Glucose mmol/L (%C)	4.24	3.92 (92%)	2.98** (70%)	2.15** (51%)	2.61** (62%)
Urea mmol/L (%C)	6.37	5.57 (87%)	5.91 (93%)	6.21 (98%)	8.41** (132%)
Cholesterol mmol/L (%C)	1.85	2.02 (110%)	2.42* (131%)	2.85* (158%)	3.06** (165%)
Albumin g/L (%C)	29.46	30.30 (103%)	30.38 (103%)	32.36** (110%)	36.56** (124%)
ALT U/L (%C)	49.40	52.20 (106%)	52.40 (106%)	56.80 (115%)	73.80* (149%)
Total bilirubin µmol/L (%C)	6.86	7.04 (103%)	6.66 (97%)	7.14 (104%)	8.62* (126%)
A/G	1.16	1.16 (100%)	1.20 (103%)	1.32** (114%)	1.52** (131%)
Bile acid µmol/L (%C)	12.68	11.76 (93%)	12.46 (98%)	12.48 (98%)	19.48** (154%)
Female					
Glucose mmol/L (%C)	5.52	4.35** (79%)	4.13** (75%)	2.66** (48%)	2.93** (53%)
Urea mmol/L (%C)	8.59	8.50 (99%)	8.61 (100%)	9.90 (85%)	10.35 (80%)
Cholesterol mmol/L (%C)	2.11	2.05 (97%)	2.71 (129%)	2.60 (123%)	3.15** (149%)
Albumin g/L (%C)	33.08	32.70 (99%)	35.00 (106%)	35.04 (106%)	36.25 (110%)
ALT U/L(%C)	50.00	56.40 (113%)	55.60 (111%)	51.60 (103%)	64.50 (29%)
Total bilirubin µmol/L (%C)	7.02	7.16 (102%)	6.94 (99%)	8.64* (123%)	9.73** (139%)
A/G	1.38	1.32 (96%)	1.42 (103%)	1.46 (106%)	1.58* (114%)
Bile acid µmol/L (%C)	11.54	13.12 (114%)	14.58 (126%)	16.50 (143%)	18.33 (159%)

* : p <0.05 ** : p <0.01

E. Sacrifice and pathology

1. Organ weights

At 8000 and 20000 ppm there were effects, many of which reflected the lower body weights in these animals, with lower absolute weights and unchanged or higher organ weights relative to body weight (generally reaching statistical significance) such as the heart, brain, kidney, liver, testes and epididymides. In males, the thymus, at 20000 ppm and prostate at 8000 and 20000 ppm were smaller than control even after adjustment for body weight.

At 3200 ppm there were no effects on organ weights in either sex that were consistent between absolute and adjusted organ weights, the statistical differences were not considered to reflect a clear effect of test item other than secondary to body weight differences.
There were no effects at 1280 ppm in either sex, a statistical difference in male kidney weights adjusted for bodyweight was within the normal range and not considered a clear test item related effect.

2. Gross and histopathology
Potentially test item-related macroscopic changes were observed at necropsy in the High dose group fed diet with 20000 ppm BYI 02960-difluoroethyl aminofuranone. These consisted of small prostate and seminal vesicles in 5/5 males and small spleen, in 2/5 males, were associated with the potential test item related changes in the terminal body weights and correlated with the organ weight changes, however, a definitive attribution cannot be made without histopathological evaluation.
Other observations were considered to be incidental or associated with morphological changes during oestrus cycle.

III. Conclusions

In conclusion, based on the effects noted in the current 14-day preliminary study and in correlation with the previous data available at the Sponsor, the dose levels selected were 200, 800 and 3000 ppm. These levels were considered to be suitable for an upcoming 28-day dietary study with BYI 02960-difluoroethyl aminofuranone.

Oral 90-day toxicity in the rat

Report:	**KIIA 5.8/14; [redacted] R.; 2012**
Title:	BYI 02960-difluoroethyl aminofuranone: A 28-day dietary toxicity study in Wistar rats
Report No & Document No	10116-100P; M-426136-01-2
Guidelines:	OECD 407 (2008); Commission Regulation EC 440/2008, Method B.7. (2008); EPA Health Effects Test Guideline (OPPTS 870.3050; 2000)
GLP	Yes (certified laboratory)

Executive Summary

BYI 02960-difluoroethyl aminofuranone, a metabolite of BYI 02960 (batch number NLL 8671-12-1: a white powder, 98.9% w/w purity) was administered continuously via the diet to groups of Wistar rats (10/sex/group) for 28 consecutive days at concentrations of 200, 800 and 3000 ppm (equating to approximately 17, 68 and 243 mg/kg body weight/day in males and 19, 76 and 273 mg/kg body weight/day in females). A similarly constituted group received untreated diet and served as a control. Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. An evaluation for any potential neuro- or ophthalmo-toxicity was conducted on Day 26. Body weight and food consumption were measured at least weekly. Prior to necropsy, the oestrous cycle of all females was evaluated by examination of vaginal smears. Clinical pathology investigations (hematology, coagulation, clinical chemistry and urinalysis) were conducted at the completion of the treatment period, before necropsy on Day 28. At termination, necropsy with macroscopic examination was performed. Weights of selected organs were recorded and representative tissues/organs were

sampled and preserved in appropriate fixatives. Full histopathology was performed on the selected list of tissues from the control and high dose animals and on any macroscopic abnormality from all animals.

There was no unscheduled mortality during the study, clinical signs, signs of neurotoxicity or any test item related, or adverse effects on the landing foot splay or grip strength under the conditions of this study. Evaluation of the vaginal smears prior to necropsy showed the expected distribution of the oestrus cycle phases within the normal population of female Wistar rats.

There was a slight but not statistically significant trend towards lower body weights at 3000 ppm in both sexes (5 - 6% below control at termination), this body weight effect corresponds with findings in a preliminary study where body weights were 15 - 20% below control at 8000 ppm. There were statistically significantly lower weight gains in both sexes in the first week of the study but the overall weight gains for the study duration were not statistically different. Other statistically significant differences were not considered to be of biological significance.

There were no test item related adverse effects on the animal food consumption during the study. No test-item related adverse effects were noted on the food conversion efficiency FCE values (g/g, calculated as weekly body weight gain g/weekly food consumption g) at any of the dose levels tested and the mean values were similar with the ones recorded in the control group.

The variations in hematological parameter compared to controls were not considered toxicologically significant or related to treatment, in the absence of a consistent dose or gender response and as the individual and mean values remained within the physiological ranges.

No significant, adverse effects were noted at evaluation of the clinical chemistry parameters evaluated at up to and including 3000 ppm. No statistically significant differences or toxicologically relevant effects were observed at urinalysis conducted at the completion of the treatment.

No test item-related macroscopic or microscopic findings were observed at the end of the study. There were no changes considered toxicologically significant in the absolute or relative organ weight values, relative to the body or brain weight, noted after BYI 02960-difluoroethyl aminofuranone administration in diet for 28 days, at up to and including 3000 ppm, evaluated immediately after completion of the treatment.

In conclusion, based on the effects noted in the current 28-day dietary study and in correlation with the previous data available at the Sponsor and experiments conducted, the No Observed Adverse Effect Level (NOAEL) under the conditions of this study is considered to be 3000 ppm equating to approximately 243 mg/kg bw/day for the males and 273 mg/kg bw/day for the females.

I. Materials and Methods

A. Material

1. Test Material:	BCS-CC98193, BYI 02960-difluoroethyl aminofuranone
Description:	White powder
Lot/Batch:	NLL 8671-12-1
Purity:	98.9%
CAS:	1134834-71-1
Stability of test compound:	Stable from 200 to 20000 ppm for at least 5 weeks (at 200ppm) or 7 weeks (at 500 and 20000 ppm) in the diet storage room (approximately 15-21 °C) and at least 14 days in the animal room (approximately 22 ± 3 °C)
2. Vehicle and/or positive control:	None
3. Test animals:	
Species:	Rat
Strain:	Crl:WI rats
Age:	7 weeks
Weight at dosing:	243 to 287 g for the males; 173 to 213 g for the females
Source:	[redacted], Germany
Acclimation period:	2 weeks
Diet:	[redacted]® SM R/M-Z+H "Autoclaved Complete Feed for rats and mice - Breeding and maintenance" (by [redacted]), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were group housed (5 animals/sex/cage)
Environmental conditions:	Temperature: 19.7 - 23.6 °C Humidity: 30 - 48% Air changes: 15 to 20 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (6 am - 6 pm).

B. Study Design

1. In life dates

03 November to 01 December, 2011 at [redacted] Hungary.

2. Animal assignment and treatment

There were 10 rats per sex per dose group. Animals were assigned to dose groups using a randomization by weight. BYI 02960-difluoroethyl aminofuranone was administered in the diet for 28 days to Wistar rats at the following doses: 0, 200, 800 and 3000 ppm (equating to approximately 17, 68 and 243 mg/kg

body weight/day in males and 19, 76 and 273 mg/kg body weight/day in females). A negative control group received plain diet.

Table 5.8-27: Study design

Test Group	Diet Concentration (ppm)	Animals assigned	
		Male	Female
1/Control	0	10	10
2/Low dose	200	10	10
3/Mid dose	800	10	10
4/ High dose	3000	10	10

Dose selection

The concentrations of BYI 02960-difluoroethyl aminofurane in [redacted] SM R/M-Z+H "Autoclavable Complete Feed for Rats and Mice - Breeding and Maintenance" were selected by the Sponsor in consultation with the Study Director based on the available data, including the results of a 14-day dose range finding and palatability study ([redacted] code 11/116-100PE) performed by the Sponsor with BYI 02960-difluoroethyl aminofuranone at [redacted] Hungary Ltd. In this 14-day range-finding study, the animals were treated with BYI 02960-difluoroethylaminofuranone at 1280, 3200, 8000 and 20000 ppm. Dose-related decreased body weight compared to control means was observed in males and females at 3200 ppm and above. Food consumption decreases occurred in males and females at 8000 and 20000 ppm. Haematological and clinical chemistry changes were also noted, including decreases in mean glucose concentrations at all dose levels in females and at 3200 ppm and above in males. These effects lead to the dose level selection in the current 28-day rat study.

3. Diet preparation and analysis

BYI 02960-difluoroethyl aminofuranone was incorporated into [redacted] SM R/M-Z+H "Autoclavable Complete Feed for Rats and Mice - Breeding and Maintenance" by [redacted] Germany and mixed for up to approximately 14 minutes (approximately 6 minutes for premix preparation, and 4 - 8 minutes for preparation of the complete diets), in a room where the temperature and humidity were controlled. Following mixing, pellets were prepared by simple compression; no binding agents, steam, external heat, any other process or substance were used that might affect BYI 02960-difluoroethyl aminofuranone or the quality of the diets. Similar diet preparation procedures were used to generate control diet (0 mg BYI 02960-difluoroethyl aminofuranone/kg diet).

The prepared diets were stored at room temperature under dry conditions, in sewed bags pending and during transport [redacted] Hungary [redacted] Hungary [redacted]., the prepared diets were stored in areas designated for diet storage at room temperature (approximately 15 - 21 °C), under dry conditions, pending transfer to animal room at approximately 22 ± 3 °C for animal feeding.

Analyses of the diets for homogeneity and/or concentration of BYI 02960-difluoroethyl aminofuranone were performed based on an HPLC analytical method using UV detection, validated at [redacted] Hungary Ltd. (11/116-316AN). Concentration and homogeneity assessment were performed at the diet arrival; additional concentration measurements were conducted at the end of the study, from the

remaining diet collected from the animal room on December 02, 2011. Two bags were received from each dose. From both bags six samples were taken: two from the top, middle and bottom area. The amount of the samples was approximately 500 g in all. At Week 4, five samples were collected from the diet in the diet storage room. The amount of the samples was approximately 50 g. At receipt of the diets the samples were homogenized and one extraction was carried out from each sample. From the first sample five replicate extractions were performed in order to test method precision. At Week 4 only the concentration of the diet was verified. The samples were mixed and five replicate extractions were carried out.

Diet samples were stored at room temperature, dry, pending analysis on the same day. No test item was detected in the Control samples. Interference in the control samples was less than 2% of the limit of quantitation. The test item was homogenously distributed in the diet. The concentration of the test item in the diet samples varied between 91% and 107% of the nominal values, within the target range of 100 ± 10% and thus these results were considered suitable for the study purposes.

4. Statistics

Evaluation was made by comparing the data for each of the Groups 2 to 4, respectively, against the control Group 1.The heterogeneity of variance between groups was checked by Bartlett's homogeneity of variance test. Where no significant heterogeneity was detected, a one-way analysis of variance was carried out. If the obtained result was positive, Duncan's Multiple Range test was used to assess the significance of inter-group differences. Where significant heterogeneity was found, the normal distribution of data was evaluated by Kolmogorov-Smirnov test.

In case of abnormal distribution, the non-parametric method of Kruskal-Wallis One-Way analysis of variance was applied. If the result was positive, the inter-group comparisons were performed using Mann-Whitney U-test. The mean and standard deviations values, the frequency of clinical observations and macroscopic findings were calculated as applicable.

C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily. All animals were observed for clinical signs at least once daily at approximately the same time, with minor variations as practical. Detailed physical examinations were performed on all animals outside the home cage at randomization (Day - 7), on the first day of treatment and at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Neurological assessment (Functional Observation Battery)

On Day 26 a.m., each animal was subjected to the functional observation battery, including qualitative assessment of the grip strength, and to measurements of the landing foot splay and fore/hind grip strength.

To measure the landing foot splay, the hind/fore paws of the rat were painted with ink and the rat were dropped from a horizontal position onto the appropriate record sheet covering the examination table. The distance between the two resulting ink spots was measured.

Fore/hind grip strength measurements were conducted using a grip strength meter (Model GS3, Bioseb, Chaville, France), an instrument designed to quantify objectively rodent muscular strength, in order to identify and assess quantitatively any potential effect of test item.
Sensory reactivity to different type of stimuli (*e.g.* auditory, visual and proprioceptive), assessment of grip strength and motor activity were conducted and the general physical condition and behavior of animals were tested. A modified Irwin test was performed. A detailed assessment for neurotoxicity effects were made on the basis of these measurements

3. Examination of vaginal smears
Prior to necropsy, the phase of the oestrus cycle of all females was determined by taking vaginal smears, which were prepared and stained with 1% aqueous methylene blue solution. The smears were examined with a light microscope, in order to provide information regarding the stage of oestrus cycle at the time of sacrifice and assist in histological evaluation of oestrogen sensitive tissues.

4. Ophthalmological evaluation
Ophthalmoscopy examination was conducted in all animals on Day - 8/- 9 (males/females) before treatment, and in the Control Group 1 and High dose Group 4 animals, towards the end of the treatment period on Day 26 pm. As no test item related changes were noted, no additional examination was considered required. Mydriasis was produced after instillation of eye drops "Mydrum" (0.5% tropicamid) into the conjunctival sac. The evaluation were performed by external examination and using a Gowlland ophthalmoscope.

5. Body weight
Body weights were recorded with a precision of 1 g at randomization (Day -7), on the first day of treatment (Day 0, prior to start of treatment), then at least weekly, including on Day 27, and prior to scheduled necropsy, fasted, on Day 28.

6. Food consumption and compound intake
Food consumption were measured pre-treatment from Day -7 to Day 0. The remaining, non-consumed food given as of Day 0 was weighed at least weekly with a precision of 1 g during the treatment period. Weekly food consumption was calculated for reporting purposes. Food conversion efficiency (g/g) was calculated as [weekly body weight gain (g)/weekly food consumption (g)].

7. Clinical pathology
Blood sampling
Blood samples for clinical pathology evaluation were collected from all animals immediately prior to the scheduled necropsy on Day 28, after an overnight period of food deprivation of animals, by heart puncture under pentobarbital anaesthesia.

Three samples were taken from each animal, one for haematology (approximately 1.2 mL blood into K_3-EDTA tubes, 1.6 mg/mL blood), one for determination of blood clotting times (approximately 1.2 mL blood for APTT and PT measurements, into sodium citrate tubes) and the third one to obtain serum samples (up to 2.6 mL blood as practical, into tubes with no anticoagulant) for clinical chemistry.

Hematology

Red blood cell count, haemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cell volume, reticulocyte count, white blood cell count and differential count evaluation and platelet count and mean platelet volume were assayed using an Advia 120.

Blood smears were prepared for all animals but they were not examined, since no test item related effects were noted in the hematology parameters evaluated.
Prothrombin time and Activated partial thromboplastin time were assayed on an AMAX Destiny Plus Coagulometer.

Clinical chemistry
Total bilirubin, glucose, urea, creatinine, total protein, albumin, total cholesterol, triglycerides, phosphorus, sodium, potassium, calcium and chloride concentrations, and γ-glutamyltransferase, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed using an VITROS equipment. Bile acids were evaluated on a Lory 2000.

Urinalysis
Urinalysis was performed during the last week of the study on Day 28. Urine samples were collected for 16 hours after an overnight food and water deprivation of animals according to the study schedule from each animal by placing the animals in metabolic cages.
Urinary volumes were determined by volumetric method. Glucose, bilirubin, ketone bodies, blood erythrocytes, leukocytes protein, urobilinogen, nitrite and pH were assayed using a Medi-test Stick 10 (Uryxxon 300). Microscopic examination of the urinary sediment was performed after centrifugation of the urine. Specific gravity was determined using a gravimetric method (Mettler PG 203-S).

8. Sacrifice and pathology
Necropsy and macroscopic examination were performed on all animals, at the end of treatment period on Day 28 (after the blood collection for clinical pathology evaluation). The animals were euthanized by exsanguination under pentobarbital anaesthesia.
After exsanguination the external appearance was examined, cranium, thoracic and abdominal cavities were opened and the appearance of the tissues and organs was observed macroscopically. Any abnormalities were recorded with details of the location, color, shape and size, as appropriate.

The following organs were weighed: Adrenal glands, brain, epipidymides, heart, kidney, liver, prostate, ovaries, seminal vesicles with coagulating glands, spleen, testes, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix). Paired organs were weighed together.
On completion of the macroscopic examination, the following organs or tissues were sampled: Adrenal gland, aorta, bone (sternum), bone marrow (sternum), brain, epididymis, oesophagus, external lachrymal gland, eye and optic nerve, harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, liver, lung with bronchi, lymph nodes (mandibular, mesenteric), mammary gland, ovary with oviduct, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle with coagulating glands, skeletal muscle (quadriceps), skin and subcutis, spinal cord (cervical, thoracic, lumbar), spleen, stomach, salivary glands (mandibular, sublingual and parotid glands), testis, thymus, thyroid gland (with parathyroid), tongue, trachea (with main stem bronchi), urinary bladder, uterus (with cervix), vagina.

The eyes with the optic nerve were retained in modified Davidson's fixative. Testes and epididymides were preserved in Bouin's solution, all other organs in 10% buffered formalin solution.
The retained tissues and organs were embedded in paraffin wax, sections were cut at 4 - 6µ by microtome and transferred to slides. Tissue sections were stained with haematoxylin-eosin/phloxine and examined by light microscope.
Full histopathology were performed in Groups 1 (Control) and 4 (High dose) and any organs or tissues with macroscopic abnormalities. Since no test item related findings were noted, no additional histopathology evaluation was required from lower dose levels.

II. Results and discussion

A. Observations

1. Mortality

There was no unscheduled mortality during the study.

2. Clinical signs of toxicity

There were no clinical signs observed during the study. No adverse effects were noted at the ophthalmoscopic evaluation conducted by the clinical veterinarian towards completion of the treatment on Day 26.
No test item related adverse effects were noted during the modified Irwin test. Vocalisation was observed throughout all dose groups including controls and was considered an expected reaction to animal handling, unrelated to treatment or to reflect an adverse effect. There were no effects considered adverse or related to test item administration at the landing foot splay or grip strength evaluation.

3. Vaginal smear evaluation

Evaluation of the vaginal smears prior to necropsy showed the expected distribution of the oestrus cycle phases within the normal population of female Wistar rats.

B. Body weight and weight gain

There were no body weight or body weight gain changes considered clearly related to BYI 02960-difluoroethyl aminofuranone administration, or to reflect an adverse effect, in any of the dose groups tested.
There was a slight but not statistically significant trend towards lower body weights at 3000 ppm in both sexes (5 - 6% below control at termination), this body weight effect corresponds with findings in a preliminary study where body weights were 15 - 20% below control at 8000 ppm.
There were statistically significantly lower weigh gains in both sexes in the first week of the study, up to approximately - 18% in the 3000 ppm males and - 38% in the females, although without a clear dose response (-31.82% lower than control mean body weight gain values were noted in the 3000 ppm females) and 33.33% higher than control mean value during the following week. The overall weight gains for the study duration were not statistically different.

Other statistically significant differences were not considered to be of biological significance, for example, there were lower gains in the 200 ppm females only, between Day 14 to Day 21, however, with no dose response, or higher than control, in the 200 and 800 ppm males between Days 7 - 14.

Table 5.8-28: Summary of body weight and body weight gain data (g)

Dose levels of Test item (ppm)	0	200	800	3000
Male				
Initial BW (Day 0) (%C)	263.4	268.9 (102%)	264.2 (100%)	260.2 (99%)
BW (Day 7) (%C)	315.3	319.1 (101%)	317.6 (101%)	302.9 (96%)
BW (Day 14) (%C)	353.0	369.9 (105%)	365.2 (103%)	337.0 (95%)
Final BW (Day 27) (%C)	412.3	437.6 (106%)	426.9 (104%)	390.8 (95%)
BWG (Day 0-7) (%C)	51.9	50.2 (97%)	53.4 (103%)	42.7* (82%)
BWG (Day 7-14) (%C)	37.7	50.8* (135%)	47.6* (126%)	34.1 (89%)
BWG (Day 14-21) (%C)	38.4	39.9 (104%)	37.4 (97%)	33.7 (88%)
Overall BWG (Days 0-27) (%C)	148.9	168.7 (113%)	162.7 (109%)	130.6 (88%)
Female				
Initial BW (Day 0) (%C)	197.2	199.4 (101%)	193.1 (98%)	192.5 (98%)
BW (Day 7) (%C)	225.8	217.4 (96%)	214.2 (95%)	212.0* (94%)
BW (Day 14) (%C)	236.9	232.2 (98%)	230.6 (97%)	224.2 (95%)
Final BW (Day 27) (%C)	262.0	251.8 (96%)	254.5 (97%)	248.7 (95%)
BWG (Day 0-7) (%C)	28.6	18.0** (63%)	21.1* (74%)	19.5** (68%)
BWG (Day 7-14) (%C)	11.1	14.8 (133%)	16.4 (148%)	12.2 (110%)
BWG (Day 14-21) (%C)	16.8	10.3* (61%)	19.1 (114%)	12.7 (76%)
Overall BWG (Days 0-27) (%C)	64.8	52.4 (81%)	61.4 (95%)	56.2 (87%)

* : p <0.05 ** : p <0.01

C. Food consumption and compound intake

There were no test item related adverse effects on the animal food consumption during the study.
No test-item related adverse effects were noted on the food conversion efficiency FCE values (g/g, calculated as weekly body weight gain g/weekly food consumption g) at any of the dose levels tested and the mean values were similar with the ones recorded in the control group.

The mean intake over the 28 days was 17, 67 and 244 mg/kg bw/day for the males and 19, 76 and 273 mg/kg bw/day for the females from Groups 2, 3 and 4, respectively.

Table 5.8-29: Summary of food consumption (g)

Dose levels of Test item (ppm)	0	200	800	3000
Male				
FC (Days 0-7) (%C)	**28.1**	**28.5 (101%)**	**28.4 (101%)**	**25.9 (92%)**
FC (Days 21-27) (%C)	**28.4**	**29.6 (104%)**	**29.6 (104%)**	**27.2 (96%)**
Female				
FC (Days 0-7) (%C)	**22.7**	**21.2 (93%)**	**21.1 (93%)**	**19.7 (87%)**
FC (Days 21-27) (%C)	**21.9**	**21.0 (96%)**	**21.1 (96%)**	**20.7 (94%)**

D. Clinic pathology

1. Hematology

When compared to the controls, there were no differences that were considered toxicologically significant noted in the treated animals. A few of the haematology parameters evaluated showed variations which on occasion attained statistical significance.

These included, for example, slightly higher RBC, HGB and HCT, monocytes MO% or PTT in the male, but not female animals, or higher WBC at 200 and 800 ppm in the males, but not at 3000 ppm, and at 800 and 3000 ppm in the females, however, with mean values comparable with the physiological ranges, or lower eosinophil EOS% in the females at all dose levels tested, but not in the males. In the absence of a consistent dose or gender response and as the individual and mean values remained within the physiological ranges, these variations were not considered toxicologically significant or related to treatment.

2. Clinical chemistry

No BYI 02960-difluoroethyl aminofuranone-related changes or toxicologically significant, adverse effects were noted at evaluation of the clinical chemistry parameters evaluated at up to and including 3000 ppm.

Statistically significant variations were noted on occasion in a few parameters. Evaluation of the mean and individual results in comparison with the control and historical control data did not reveal any test-item related cause of the changes noted, and/or no consistent dose or gender-related response was observed. Therefore, these differences observed between the control and treated groups were considered to be incidental or individual findings, which were not related to treatment, were generally minor and remained within the historical control ranges or were with no toxicological significance.

Table 5.8-30: Mean glucose concentrations (% to control mean)

Dose levels of Test item (ppm)	0	200	800	3000
Male				
Glucose mmol/L (%C)	**6.497**	**7.403 (114%)**	**5.583 (86%)**	**5.846 (90%)**
Female				
Glucose mmol/L (%C)	**7.216**	**7.686 (106%)**	**6.900 (96%)**	**5.671 (89%)**

3. Urinalysis

No statistically significant differences or toxicologically relevant effects were observed at urinalysis conducted at the completion of the treatment.

E. Sacrifice and pathology

1. Macroscopic findings

There was no evidence of the test item-related observations at necropsy.

Incidental changes including bilateral enlargement of the adrenals, dilatation of the renal pelvis and enlargement and/or discoloration of the prostate were noted with low incidence and/or throughout all the dose groups, unrelated to treatment.

2. Organ weights

There were no changes considered toxicologically significant in the absolute or relative organ weight values, relative to the body or brain weight, noted after BYI 02960-difluoroethyl aminofuranone administration in diet for 28 days, at up to and including 3000 ppm, evaluated immediately after completion of the treatment.
No statistically significant differences were recorded at evaluation of the absolute mean organ weights. Variations were observed in the relative organ weights, on occasion attaining statistical significance, including, for example, slightly higher kidney weight adjusted for the terminal body weight in the 3000 ppm male and female animals, but not when adjusted for the brain weight, slightly higher testes and seminal vehicles weights relative to the terminal body weight in the 3000 ppm males, or slightly higher thymus (males), or liver (females) weight relative to the brain weight in the 800 ppm animals , but not in the other gender, and not at 3000 ppm. These values were within the normal range, or the variations had low magnitude, showed no consistent response between genders, and/or were not correlated with pathological findings, they were considered incidental and not related to BYI 02960-difluoroethyl aminofuranone administration.

3. Gross and histopathology
No effects of the test item were noted microscopically.
The microscopic changes in the kidneys, liver and prostate were regarded as incidental.
The splenic hematopoiesis was considered to be common background observation.
Minimal to mild extramedullary hematopoiesis in the spleen was present in 14/20 Control and High Dose 18/20 rats.
In the prostate, mild cellular debris or neutrophilic infiltration and moderate necrotizing inflammation were recorded.
In the liver, minimal or mild focal hepatocellular necrosis was noted in 2/10 High Dose males. No hepatic microscopic changes were observed in High Dose females.
Additionally, minimal focal tubular basophilia or minimal to mild pelvic dilatation of the right kidney, were also sporadically seen.

III. Conclusions

In conclusion, , the NOAEL under the conditions of this study is considered to be 3000 ppm, equating to approximately 244 mg/kg bw/day for the males and 273 mg/kg bw/day for the females.

BYI 02960-CHMP (6-chloropyridin-3-ylmethanol)

In vitro *genotoxicity - Bacterial assay for gene mutation*

Report:	**KIIA 5.8/15, [redacted], 1994 amended in 1997**
Title:	IM-0 – Reverse mutation study on bacteria
Report No & Document No MRID No.	G-949 M-195904-01-2 44988432 (submitted by Nippon Soda Co., Ltd., JPN)
Guidelines:	OECD 471 (1997); EPA Health Effects Test Guideline 84-2, JMAFF guideline 59 Nohsan N° 4200
GLP	Yes (certified laboratory)

Executive Summary

The mutagenicity of IM-0, ((6-chloro-3-pyridyl)methanol, Lot No. NK-3120, Purity 99.14%) was examined by the reverse mutation study using 4 strains (TA100, TA1535, TA98 TA1537) of *Salmonella typhimurium* and 1 strain (WP2 uvrA) of *Escherichia coli.* The study was performed with pre-incubation in both of the method with and without metabolic activation by S9Mix (Experimental No. 9862-1, 9862-2). As a result, IM-0 didn't increase in the number of reverse mutant colonies regardless of the presence or absence of the metabolic activation.
Therefore, we conclude that the mutagenicity of EVI-0 was negative under these experimental conditions.

I. Materials and Methods

A. Material

1. Test Material:	IM-0,(6-chloro-3-pyridyl)methanol
Description:	White crystal
Lot/Batch:	NK-3120
Purity:	99.14%
CAS:	21543-49-7
Stability of test compound:	5% aqueous solution stable for 4 hours at 24 °C
2. Control materials:	Negative: Culture medium
	Solvent: DMSO for reference compounds, water for BYI 02960-CHMP
	Positive: N-ethyl-N'-nitro-N-nitrosoguanidine for TA 1535 (at 5 µg/plate), TA 100 at 3 µg/plate and WP2 uvrA at 2 µg/plate, 2-Nitrofluorene for TA 98 at 0.2 µg/plate, 9-Aminoacridine hydrochloride for TA 1537 at 80 µg/plate, 2-Aminoanthracene for the activating effect of the S9 mix in TA98 at 0.5 µg/plate, in TA 100 at 1 µg/plate, in TA 1535 and TA 1537 at 2 µg/plate and in WP2 uvrA at 10 µg/plate

3. Test organisms:

Species:	Salmonella typhimurium LT2 mutants and Escherichia coli
Strain:	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102 and Escherichia coli WP2 uvrA strain
Source:	Strains obtained from [redacted]

4. Test compound concentrations:

Experiment I and II:	For all strains with or without S9 mix: 313, 625, 1250, 2500 and 5000 µg/plate

B. Study Design and methods

The experimental phase of the study was performed between November 19 to December 6, 1993 at [redacted]

The Salmonella/microsome test is a screening method which detects point mutations caused by chemical agents *in vitro*. Auxotrophic mutants of *Salmonella typhimurium* are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

Three test tubes and three minimum glucose agar plates were prepared for each test concentration and each of the solvent control and the positive controls. S9 mix was prepared from the liver of Sprague Dawley rats treated with 5,6-benzoflavone at 80 mg/kg for one day and phenobarbital at 30-60 mg/kg for 4 days intraperitoneally. The agar solution was complemented with 0.5 mM histidine and 0.5 mM biotin for *Salmonella typhimurium* and with 0.5 mM tryptophan for *Escherichia coli*.
In a preliminary test, no growth inhibition was observed at 2 500 mg/plate, the highest concentration tested. The highest concentration of the main experiment was set up at 5 000 µg/plate.
0.1mL of the test item solution, 0.5 mL of 0.1M sodium phosphate buffer or 0.5 mL of S9 mix for the metabolic activation method and 0.1 mL of the bacterial culture were put into a test tube and mixed. The mixture was incubated for 20 minutes at 37 °C under shaking. Then, 2 mL of the top agar was added to the tube. They were stirred and poured onto the agar plate. After the agar hardened, the plate was moved in the incubator and cultures at 37 °C.
The number of reverse mutant colonies was measured with the naked eyes, with the stereoscopic microscope or with the automatic colony counter. The presence of absence of the growth inhibition and the precipitate were observed with the stereoscopic microscope.

No statistical method was used. The test substance was characterized as positive in the test that fulfilled the following requirements: doubling of the spontaneous mutation rate of the solvent control, dose-response relationship, and reproducibility of the results.

II. Results and discussion

In the first main experiment, no increase in the number of reverse mutant colonies was observed in any strain, either in the presence or absence of the metabolic activation. Precipitation of the test substance was observed at the highest concentration.
In the second main experiment, neither increase in the number of reverse mutant colonies nor growth inhibition was observed in any strain in the presence or absence of the metabolic activation. There was no precipitation of the test substance even at the highest concentration.

Table 5.8-23: Mean numbers of revertant colonies ± SD in the first experiment

Test item	Concentration (µg/plate)	TA100	TA1535	TA98	TA1537	WP2 uvrA
Without S9 mix						
Control	**-**	**135 ± 5.03**	**9 ± 4.04**	**29 ± 8.19**	**7 ± 4.73**	**30 ± 4.36**
Test item	**313**	**124 ± 8.54**	**15 ± 3.77**	**21 ± 3.0**	**5 ± 3.06**	**25 ± 7.02**
	625	**139 ± 8.96**	**14 ± 3.46**	**34 ± 4.51**	**6 ± 2.08**	**28 ± 2.52**
	1250	**145 ± 10.0**	**13 ± 5.29**	**31 ± 5.69**	**3 ± 0.58**	**28 ± 6.56**
	2500	**140 ± 5.86**	**9 ± 3.06**	**30 ± 4.62**	**9 ± 4.04**	**26 ± 7.55**
	5000	**141 ± 3.51**	**10 ± 2.65**	**26 ± 8.08**	**6 ± 1.15**	**30 ± 6.81**
ENNG	**3**	**549 ± 34.04**	**-**	**-**	**-**	**-**
ENNG	**5**	**-**	**463 ± 18.73**	**-**	**-**	**-**
2NF	**0.2**	**-**	**-**	**89 ± 4.36**	**-**	**-**
9AA	**80**	**-**	**-**	**-**	**528 ± 16.65**	**-**
ENNG	**2**	**-**	**-**	**-**	**-**	**380 ± 32.08**
With S9 mix						
Control	**-**	**132 ± 9.85**	**10 ± 3.21**	**29 ± 6.24**	**14 ± 4.93**	**32 ± 3.61**
Test item	**313**	**140 ± 8.96**	**8 ± 4.51**	**37 ± 7.0**	**7 ± 3.51**	**40 ±6.0**
	625	**147 ± 22.03**	**9 ± 2.08**	**36 ± 6.43**	**15 ± 3.79**	**34 ± 7.64**
	1250	**135 ± 10.54**	**14 ± 2.08**	**43 ± 3.21**	**16 ± 2.08**	**32 ± 14.01**
	2500	**146 ± 2.0**	**15 ± 2.31**	**33 ± 6.03**	**17 ± 4.93**	**28 ± 2.65**
	5000	**149 ± 2.89**	**13 ± 3.79**	**40 ± 8.96**	**13 ± 0.58**	**29 ± 7.21**
2AA	**1**	**560 ± 18.73**	**-**	**-**	**-**	**-**
2AA	**2**	**-**	**197 ± 16.17**	**-**	**158 ± 7.64**	**-**
2AA	**0.5**	**-**	**-**	**174 ± 9.29**	**-**	**-**
2AA	**10**	**-**	**-**	**-**	**-**	**694 ± 10.0**

Table 5.8-24: Mean numbers of revertant colonies ± SD in the second experiment

Test item	Concentration (µg/plate)	TA100	TA1535	TA98	TA1537	WP2 uvrA
Without S9 mix						
Control	**-**	**134 ± 7.02**	**10 ± 3.0**	**28 ± 12.42**	**6 ± 1.0**	**17 ± 3.61**
Test item	**313**	**142 ± 5.29**	**6 ± 1.53**	**23 ± 4.36**	**5 ± 2.08**	**14 ± 2.31**
	625	**128 ± 3.21**	**6 ± 1.73**	**24 ± 3.61**	**4 ± 1.15**	**18 ± 2.08**
	1250	**131 ± 4.36**	**8 ± 3.79**	**22 ± 1.73**	**7 ± 2.65**	**19 ± 2.89**
	2500	**135 ± 6.81**	**9 ± 3.21**	**23 ± 4.04**	**7 ± 1.53**	**14 ± 3.21**
	5000	**136 ± 11.15**	**11 ± 2.08**	**19 ± 4.93**	**5 ± 1.53**	**18 ± 5.03**
ENNG	**3**	**517 ± 9.54**	**-**	**-**	**-**	**-**
ENNG	**5**	**-**	**489 ± 64.65**	**-**	**-**	**-**
2NF	**0.2**	**-**	**-**	**87 ± 2.52**	**-**	**-**
9AA	**80**	**-**	**-**	**-**	**615 ± 8.19**	**-**
ENNG	**2**	**-**	**-**	**-**	**-**	**301 ± 10.69**
With S9 mix						
Control	**-**	**125 ± 5.57**	**10 ± 1.53**	**46 ± 3.21**	**13 ± 4.04**	**26 ± 1.0**
Test item	**313**	**133 ± 11.02**	**10 ± 3.51**	**38 ± 6.66**	**16 ± 3.61**	**18 ± 2.52**
	625	**110 ± 24.21**	**8 ± 4.0**	**44 ± 3.61**	**21 ± 1.53**	**23 ± 2.65**
	1250	**115 ± 9.54**	**14 ± 6.66**	**44 ± 2.08**	**15 ± 3.61**	**21 ± 0.58**
	2500	**129 ± 13.61**	**6 ± 1.0**	**45 ± 2.65**	**11 ± 2.52**	**18 ± 3.06**
	5000	**144 ± 3.79**	**7 ± 2.08**	**46 ± 3.61**	**18 ± 7.64**	**14 ± 4.58**
2AA	**1**	**563 ± 2.08**	**-**	**-**	**-**	**-**
2AA	**2**	**-**	**203 ± 24.44**	**-**	**144 ± 18.45**	**-**
2AA	**0.5**	**-**	**-**	**113 ± 8.50**	**-**	**-**
2AA	**10**	**-**	**-**	**-**	**-**	**574 ± 28.02**

III. Conclusions

No indication of mutagenic effects of BYI 02960-CHMP (metabolite of BYI 02960) was found at assessable doses of up to 5000 µg/plate in any of the *Salmonella typhimurium* strains used in the assay or the *Escherichia coli* strain.

Acute oral toxicity

Report:	**KIIA 5.8/16, [redacted] 1993 amended in 1997**
Title:	IM-0, Acute oral toxicity in the rats
Report No & Document No MRID No:	G-0887 M-195899-01-2 44988421 (submitted by Nippon Soda Co., Ltd., JPN)
Guidelines:	OECD 401,); EPA Health Effects Test Guideline 81-1, JMAFF guideline 59 Nohsan No 4200
GLP	Yes (certified laboratory)

Executive Summary

An acute oral toxicity study was performed with IM-0 ((6-chloro-3-pyridyl)methanol, batch N° NK-2327'-6, 98.65% of purity) administered at 1000, 1500, 2000 and 3000 mg/kg to 7 weeks old Sprague Dawley rats (5/sex/group). An additional group of five females received the test item at 1300 mg/kg. Clinical signs and mortality were observed for 14 days. Body weight was measured on days 0, 1, 2, 3, 7 and 14. A gross necropsy was performed on all animals that died during the observation period and on all animals that survived until the termination of the experiment.
All animals administered 3000 mg/kg died. 100% mortality was also observed in the females treated at 2000 mg/kg, whereas only 60% mortality was observed in the males at the same dose level. 60% mortality was observed in the females treated at 1500 mg/kg and 20% in the males. No mortality was observed at 1000 mg/kg in both sexes and at 1300 mg/kg in females.
An absence of righting reflex and a decrease in motor activity, hypotonea, prone position and ataxia were observed at all doses in both sexes. All signs disappeared on the day after administration for the males and on day 2 for the females. No body weight effects were observed in males. A slight body weight loss was observed in 3/5 females treated at 1300 mg/kg and 1/3 females at 1500 mg/kg one day after administration. The animals from these groups recovered thereafter. Gastric haemorrhagies were observed in one male and one female treated at 1500 mg/kg.

The calculated LD_{50} values (Probit method) were:
Male: 1842 mg/kg (95% confidence limits: 1389 - 2622 mg/kg)
Female: 1483 mg/kg.

I. Materials and Methods

1. Test Material: IM-0, 6-chloropyridin-3-ylmethanol
Description: Pale yellow crystal
Lot/Batch: NK-2327'-6
Purity: 98.65%
CAS: 21543-49-7
Stability of test compound: No analysis performed during the study

2. Vehicle and /or positive control: Ion-exchange water

3. Test animals:

Species:	Rat
Strain:	Crj:CD (SD), SPF
Age:	7 weeks
Weight at dosing:	200.8 to 222.2 g in males and 140.1 to 166.8 in females
Source:	[redacted] Japan Inc.
Acclimation period:	7 days
Diet:	Pelleted diet, MF® (Oriental Yeast Co., Ltd), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were group caged conventionally in stainless steel mesh cages
Environmental conditions:	Temperature: 21.7 ± 0.3 °C
	Humidity: 67.3 ± 0.9%
	Air changes: Approximately 12 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates

March 22 to April 21, 1993 performed at Toxicology Laboratory of [redacted]

2. Animal assignment and treatment

The substance was tested at 1000, 1500, 2000 and 3000 mg/kg in five male and five female Sprague Dawley rats per group plus an additional group of five females treated at 1300 mg/kg. The animals were randomly allocated to cages. Following an overnight fast, the animals received a single dose of IM-0 (6-chloropyridin-3-ylmethanol, BYI02960-CHMP) by gavage. The test substance was administered in ion-exchange at a volume of 10 ml/kg bw. Clinical signs and mortality rates were determined several times on the day of administration and subsequently at least once daily for an observation period of at least 14 days. Body weights were recorded prior to administration and on days 1, 2, 3, 7 and 14 and at death except for animals that died on day 0. A gross necropsy was performed on all animals that died during the observation period and on all animals that survived until the termination of the experiment.

3. Calculation of LD50 value

Based on mortalities on Day 14, LD_{50} value was calculated by Probit method.

II. Results and discussion

A. Mortality

All deaths occurred within 1or 2 days after the administration in males and females, respectively. The mortality rate is shown in the following table.
The acute oral LD_{50} values for IM-0 were 1,842 mg/kg (95% confidence limits: 1389 - 2622 mg/kg) for males and 1483 mg/kg for females.

Table 5.8-25: Mortality rate in rats administered with BYI 02960-CHMP

Dose levels in mg/kg	Mortalities (%) in male	Mortalities (%) in female
1000	0	0
1300	Not tested	0
1500	20	60
2000	60	100
3000	100	100

B. Clinical observations

The absence of righting reflex and a decrease in motor activity, hypotonea, prone position and ataxia were observed at all doses in both sexes. All signs disappeared on the next day of the application for the males and on day 2 for the females.

C. Body weight

No body weight effects were observed in males. A slight body weight loss was observed in 3/5 females treated at 1300 mg/kg and 1/3 females at 1500 mg/kg one day after the administration. The animals from these groups recovered thereafter.

D. Necropsy

Gastric haemorrhagies were observed in one male and one female treated at 1500 mg/kg.

III. Conclusions

Under the experimental conditions of this study, the acute oral LD_{50} values for IM-0 are 1,842 mg/kg for males and 1,483 mg/kg for females, qualifying for EPA toxicity category II.

Oral 14-day toxicity in the rat

Report:	**KIIA 5.8/17, [redacted] S.; 1993 amended in 1997**
Title:	IM-0, Thirteen-week dietary subchronic toxicity study in rats
Report No & Document No MRID No:	G-0889; M-195901-01-2 44988427 (submitted by Nippon Soda Co., Ltd., JPN)
Guidelines:	OECD 408 (1998); EPA Health Effects Test Guideline 82-1; JMAFF guideline 59 Nohsan N° 4200 (1985)
GLP	Yes (certified laboratory)

Executive Summary

BYI 02960-CHMP (IM-0 or , 6-chloropyridin-3-ylmethanol) (batch number NK-3266 (Tox-563), a pale yellow crystal, 98.94% purity), a metabolite of the insecticide BYI 02960, was administered continuously via dietary administration to separate groups of Sprague Dawley rats (10/sex/group) at concentrations of 160, 800, 4000 and 20 000 ppm (corresponding to 9.9, 48.9, 250.1 and 1246.6 mg/kg/day for males and 11.1, 55.9, 276.9 and 1173.7 mg/kg/day for the females) for at least 90 days. A similarly constituted group of 10 males and 10 females received untreated diet and acted as a control. Clinical signs were recorded daily and body weight and food consumption were measured weekly. A detailed physical examination was performed weekly throughout the study. Ophthalmological examinations were performed on the animals from the control group and the high dose group during the acclimatization phase and during Week 12. Urine samples were collected overnight during the week before necropsy from all animals. Before necropsy a blood sample was collected from the carotid artery of each animal for haematology and clinical chemistry investigations. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically.

There were no signs of reaction to treatment in any treated group. All animals survived throughout the study.

Mean body weights for high dose (20 000 ppm) males and females were significantly less than respective control groups throughout the study. Mean body weights of the high dose groups were 78% of control values in males, and 77% in females. Mean body weight gains of the high dose groups were 67% of control values in males, and 57% in females.

Food consumption values of these groups were lower than control values at weeks 1-4, 6, 9, 13 (days 7 - 28, 42, 63, 91) examinations in males, and at all weeks in females. Food efficiency values of the 20 000 ppm animals were significantly decreased at weeks 1 and 10 (days 7 and 70) in males, and at week 1 (day 7) in females.

No treatment-related effects were observed at ophthalmological, haematological or urinalysis evaluations. A statistically significant increase in serum alkaline phosphatase activity was seen only in the 20000 ppm group females at study termination.

Statistically significant decreases in mean absolute weights of lung (male only) and liver (male only), and statistically significant increases in mean relative organ weight ratios of brain (both sexes), lung (females only), liver (females only), kidney (both sexes) and testis (right side only) were seen only in 20000 ppm groups. However, these changes were attributed to the decreased body weights in these groups (reduction rates, 21% in males, 22% in females).

Necropsy revealed no compound-related lesions. Histologically, dose-related eosinophilic intranuclear inclusions were seen in the proximal tubular epithelium of kidneys for 20000 ppm males and females and 4000 ppm males. Other microscopic changes were occasionally seen in the control and treated groups, but they were considered unrelated to treatment with test compound.

Based on the results mentioned above, the effects of the test item (IM-0), when administered in the diet to Sprague Dawley rats for 13 weeks, were decreased body weight gains, decreased food consumption values, increased serum alkaline phosphatase activity, and eosinophilic intranuclear inclusions in the proximal tubular epithelium of kidney. The NOAEL is 800 ppm (48.9 mg/kg/day) in males, and 4000 ppm (275.9 mg/kg/day) in females.

I. Materials and Methods

A. Material

1. Test Material: IM-0, 6-chloropyridin-3-ylmethanol
Description: Pale yellow crystal
Lot/Batch: NK-3266 (Tox-563)
Purity: 98.94%
CAS: 21543-49-7
Stability of test compound: Stable in rodent diet at 92 ppm for 4 days at room temperature and at 100 ppm for 35 days under frozen conditions

2. Vehicle and /or positive control: None

3. Test animals:
Species: Rat
Strain: Crj:CD™(SD)
Age: 5 weeks
Weight at dosing: 180.5 to 207.3 g for the males - 137.2 to 163.9 g for the females
Source: [redacted], Japan)
Acclimation period: 7 days
Diet: Powdered basal diet (MF, Oriental Yeast Co., Ltd., Tokyo), *ad libitum*
Water: Tap water, *ad libitum*
Housing: Animals were individually housed in suspended stainless steel wire mesh cages
Environmental conditions: Temperature: 22.4 ± 0.6 °C
Humidity: 60.7 ± 1.8%
Air changes: Approximately 10 to 20 changes per hour
Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates

June 30 to October 15, 1993 performed at [redacted]

2. Animal assignment and treatment

There were 10 animals of each sex per dose group. Animals were assigned to dose groups using a randomization by weight. BYI 02960-CHMP (IM-0) was administered in the diet for at least 90 days to Sprague Dawley rats at the following doses - 0, 160, 800, 4 000 and 20 000 ppm (corresponding to 9.9, 48.9, 250.1 and 1246.6 mg/kg/day for males and 11.1, 55.9, 275.9 and 1173.7 mg/kg/day for the females). A negative control group received plain diet.

3. Diet preparation and analysis

IM-0 was incorporated into the diet by dry mixing to provide the required concentrations. There were three preparations of each concentration for the whole study. The stability was demonstrated at 100 ppm after 35 days under frozen conditions and at 92 ppm after 4 days at room temperature. The homogeneity and the concentration of the diet were verified for all preparations and were within a range of 91 to 104 % of the nominal concentrations.

Table 5.8-26: Study design

Test group	Concentration in diet (ppm)	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	10	10
2	160	9.9	11.1	10	10
3	800	48.9	55.9	10	10
4	4000	250.1	275.9	10	10
5	20 000	1246.6	1173.7	10	10

4. Statistics

Statistical evaluation was made using the following methods: Chi-square test for ophthalmological observations, semi-quantitative urinalysis values (including sediment), macroscopic and microscopic observations; multiple comparison procedure ([redacted] et al. 1982) for body weights, food consumption, haematological values, biochemistry values, quantitative urinalysis values and organ weights. For multiple comparisons, Bartlett's test was used to compare the variances among groups at 5% risk level. If the variances were equal, parametric procedures were used; if not, nonparametric procedure were used. The parametric procedures were the standard one way ANOVA using the F distribution to assess significance. If a significant difference among the means was indicated, Dunnett's test (groups with equal number of data) or Scheffe's test (groups with unequal number of data) were used to determine which means were significantly different from control. The nonparametric procedures were Kruskal-Wallis test to assess significance. If a significant difference among the means was indicated, Dunnett's type test (groups with equal number of data) or Scheffe's type test (groups with unequal number of data) were used to determine which means were significantly different from control.

B. Methods

1. Observations
All rats were observed once a day for signs of overt toxicity at the times when mortality or morbundity were checked. Detailed physical examinations were conducted on all animals at least once weekly.

2. Body weight
Body weights were recorded on the first day of test substance administration, then at weekly intervals throughout the treatment.

3. Food consumption
During the treatment period, individual daily food consumption values (g/animal/day) were calculated weekly by measurements of the amount of food given and that remaining in the food hoppers.
Individual daily food consumption (g/kg/day), food efficiency (%) and compound consumption values (mg/kg/day) were calculated as follows:

Food Consumption Value (g/kg/day) = [Food Consumption Value (g/animal/day))/[Body Weight (kg)]

Food Efficiency Value (%) = [Body Weight Gain (g/animal/day)]/[Food Consumption Value (g/animal/day)] x 100

Compound Consumption Value (mg/kg/day) = [Food Consumption Value (g/animal/day)] x [Concentration(ppm)]/[Body Weight (g)]

4. Ophthalmic examination
Ophthalmological examinations (Portable slit lamp SL-14, Kowa Co., Ltd., Tokyo) were performed on all rats in the control and high dose (20,000 ppm) at study initiation and at week 12 of the study (days 84 - 85). Fundus camera (RC-2, Kowa Co., Ltd., Tokyo) was also utilized for the evaluation of fundus oculi. Mydrin ™-P (Santen Pharmaceutical Co., Ltd., Osaka) was used for pupillary dilatation.

5. Clinical chemistry
Blood sampling
At study termination (samplings were performed on days 92-94, and results are represented in tables as data at week 14), all survivors were fasted for 16 hours and more, and blood samples were collected from the carotid artery under anesthesia with intraperitoneal injection of pentobarbital sodium (Nembutal ™, [redacted], U.S.A.). EDTA-K2 and 3.8% sodium citrate solution were used as anticoagulants.

Haematology
The following haematology parameters were assayed: red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, white blood cell count and platelet count. A blood smear was prepared and stained with May-Grünwald-Giemsa and examined microscopically for differential leukocyte counts.

Biochemistry
Blood samples were obtained as mentioned previously. Sera were obtained by using centrifuge (3000 r.p.m, 15 min.) and examined in respect of the parameters mentioned below. Sodium, potassium and chloride were analyzed by IT-3 (Jookoo Co., Ltd., Tokyo) and other parameters by Centrifichem Encore ™ (Baker Instruments Co., U.S.A.).

Total bilirubin, glucose, urea, creatinine, total cholesterol, total protein, albumin, chloride, sodium, potassium, calcium and phosphorus concentrations and alkaline phosphatase and gamma-glutamyltransferase, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase and cholinesterase activities were assayed. Globulin and albumin/globulin ratio values were calculated.

6. Urinalysis
Urine samples were collected during a 24-hour fasting period from all rats housed individually in metabolism cages (MT-R, Tokyo Giken Service Co., Ltd. Tokyo) at week 13 (samplings were performed on days 85-87). During urine collection, tap water was available ad libitum.
Urinalysis was performed on the following parameters:
appearance (macroscopically), volume (graduated tube), specific gravity (Refractometer Uricon-S, Atago Co., Ltd., Tokyo), pH (pH meter HM-20E, Toa Electronics Ltd., Tokyo), water consumption (calculated by measurements of the amount of water given and that remaining in the bottles).
protein, glucose, ketone body, bilirubin, occult blood and urobilinogen. Multistix ™ SG-L (Miles-Sankyo Co., Ltd., Tokyo) was dipped in urine and analyzed by Clinitec™ 100 (Miles Inc., U.S.A.).
sediment (examined microscopically for urine collected for 3 to 6 hours).

7. Sacrifice and pathology
A complete necropsy was conducted on all remaining animals at study termination. All animals were weighed after a 16-hour fasting period and exsanguinated from the carotid artery under anesthesia with pentobarbital sodium (Nembutal™, [redacted] Laboratories, U.S.A.). After external observations, the animals were examined for cervical, abdominal, thoracic, pelvic and cranial cavities and their organs and tissues. Protocol designated organs were weighted, and their organ/body weight ratios (%) were calculated. The organs listed in the protocol were dissected and stored in 10% phosphate-buffered neutral formalin. The testes and eyes were fixed in Bouin's solution and glutaraldehyde solution, respectively, and stored in 10% phosphate-buffered neutral formalin.

Organ Weights: brain, thymus, lung, liver, spleen, kidney, adrenal, testis and ovary.

Sampling : brain, pituitary grand, eye, Harderian gland, extraorbital lacrimal gland, salivary gland (sublingual, parotid, submaxillary), submaxillary lymph node, thyroid gland, parathyroid gland, sternum with bone marrow, thymus, lung, heart, esophagus, trachea, aorta, liver, kidney, spleen, pancreas, adrenal, stomach, duodenum, jejunum, ileum, cecum, colon, mesenteric lymph node, rectum, testis, epididymis, prostate, seminal vesicle, ovary, oviduct, uterus, vagina, urinary bladder, sciatic nerve, femoral muscle, skin, mammary gland, spinal cord (cervical, thoracic, lumbar), femur, knee joint and abnormal organs or tissues.

Paraffin sections from the organs listed below were stained with hematoxylin-eosin (the production of specimen was entrusted to Hist Science Laboratory Co., Tokyo), and examined microscopically.

- All organs/tissues mentioned above from the all animals of 20000 ppm and control groups
- Organs and tissues showing macroscopic abnormalities
- Target organs from all animals
- Lungs, livers and kidneys from all animals.

In addition, the kidneys of two males, one from control group (Animal No. 0001) and the other from 20000 ppm group (Animal No. 4001), were subjected to microscopic and electronmicroscopic examination in an attempt to confirm the character of eosinophilic intranuclear inclusions seen in the proximal tubular epithelium of kidneys for 20000 ppm group males and females. For microscopic examinations, paraffin sections from kidneys were stained with Feulgen, methyl green-pyronin, periodic acid-Schiff and Ziehl-Neelsen stain.

II. Results and discussion

A. Observations

There were no mortalities during the study. Abnormal teeth, alopecia and excoriation in males, and alopecia, reddening in auricles, crust, lacrimation and abnormal teeth in females were sporadically seen in all groups, and the incidences were not treatment-related.

B. Body weight and body weight gain

Body weights for the 20000 ppm group males and females were significantly decreased throughout the study when compared with the control. Mean body weights of the 20000 ppm groups at study termination were 78% of control values in males, and 76% in females, respectively. In the 20000 ppm groups, mean cumulative body weight gains (weeks 0 - 13) were 67% of control values in males, and 57% in females, respectively.

Table 5.8-27: Group mean body weight (BW) and cumulative body weight gain (BWG) (g)

IM-0 (ppm)	0	160	800	4000	20000
Male					
Initial BW (Day 0) (%C)	193	196 (102)	199(103)	197 (102)	193 (100)
BW Week 1 (Day 7) (%C)	268	270 (101)	274 (102)	271 (101)	214** (80)
BW Week 4 (Day 28) (%C)	421	419 (100)	437 (104)	432 (103)	326** (77)
BW Week 8 (Day 56) (%C)	525	525 (100)	557 (106)	541 (103)	412** (78)
Final BW Week 13 (Day 91) (%C)	585	578 (98)	623 (106)	613 (105)	455** (78)
BWG Weeks 1-4 (Days 0 to 28) (%C)	227	223 (98)	239 (105)	235 (104)	133** (59)
BWG Weeks 1-8 (Days 0 to 56) (%C)	331	329 (100)	359 (108)	344 (104)	219** (66)
Overall BWG (Days 0 to 91) (%C)	392	382 (97)	424 (108)	417 (106)	263** (67)
Female					
Initial BW (Day 0) (%C)	149	150 (101)	151 (101)	150 (101)	147 (99)
BW Week 1 (Day 7) (%C)	179	177 (99)	177 (99)	175 (97)	150** (84)
BW Week 4 (Day 28) (%C)	243	234 (96)	243 (100)	233 (96)	191** (79)
BW Week 8 (Day 56) (%C)	292	273 (94)	290 (99)	276 (95)	225** (77)
Final BW Week 13 (Day 91) (%C)	318	301 (95)	318 (100)	301 (95)	243** (76)
BWG Weeks 1-4 (Days 0 to 28) (%C)	94	84 (90)	92 (98)	83 (88)	43.9** (47)
BWG Weeks 1-8 (Days 0 to 56) (%C)	143	123 (86)	139 (98)	126 (88)	78** (55)
Overall BWG (Days 0 to 91) (%C)	169	151 (89)	167 (99)	151 (89)	96** (57)

** : p <0.01

C. Food consumption and compound intake

Mean food consumption values (g/animal/day) of 20000 ppm males and females were decreased throughout the study when compared with the control and attained statistical significance at weeks 1 - 4, 6, 9 and 13 in males and at every week in females.

Mean food consumption values (g/kg/day) of 20000 ppm males were significantly decreased at the early phase of treatment (at weeks 1 - 2) but increased over the control group values at the latter phase of treatment (at weeks 7 - 8 and 12) while that of females in this group was significantly decreased at weeks 1, 2,4,8, 9 and 12.

Food efficiency values (%) of 20000 ppm groups were significantly inferior to that of control at early (at week 1) and later (at week 10) phase of treatment in males, and at early phase of treatment (at week 1) in females.

Table 5.8-28: Group mean food consumption (FC) (g)

IM-0 (ppm)	0	160	800	4000	20000
Male					
FC Week 1 (Day 7) (%C)	26	26 (100)	27 (104)	29 (112)	15** (58)
FC Week 4 (Day 28) (%C)	29	29 (100)	30 (103)	30 (103)	22** (76)
FC Week 8 (Day 56) (%C)	27	29 (107)	29 (107)	29 (107)	26 (96)
FC week 13 (Day 91) (%C)	26	27 (104)	28 (108)	28 (108)	22** (85)
Female					
FC Week 1 (Day 7) (%C)	18	17 (94)	16 (89)	17 (94)	12** (67)
FC Week 4 (Day 28) (%C)	19	19 (100)	20 (105)	18 (95)	12** (63)
FC Week 8 (Day 56) (%C)	19	18 (95)	18 (95)	17 (89)	11** (58)
FC week 13 (Day 91) (%C)	18	16 (89)	18 (100)	17 (94)	13** (72)

D. Achieved dosages

The mean achieved dosage intake of IM-0 per group was as follows:

Table 5.8-29: Mean achieved dietary intake of IM-0 (Weeks 1 - 13)

Diet concentration (ppm)	Male mg/kg/day	Female mg/kg/day
160	9.9	11.1
800	48.9	55.9
4000	250.1	275.9
20000	1246.6	1173.7

E. Ophthalmoscopic examination

No treatment-related ocular abnormalities were observed at ophthalmoscopic examination.

F. Blood analysis

Haematological findings

Statistically significant decreases in platelet count values ($p < 0.05$) were seen in the 160 ppm group males, but not in the 20000 ppm males. This effect was therefore not considered toxicologically relevant.

All other hematological parameters were not statistically significant different in any male or female group.

Clinical chemistry findings

The significant increases in serum potassium concentration ($p < 0.05$) and alkaline phosphatase activity ($p < 0.01$) were seen only in females receiving 20000 ppm. However, the degree of increase in

potassium concentration was slight (13% of the control value). A significant increase of serum glutamic-pyruvic transaminase ($p < 0.05$) activity was found in the 4000 ppm group males, but these were not found in the 20000 ppm group males. This effect was therefore not considered toxicologically relevant.
Any other biochemical findings were devoid of statistically significant differences in all male or female groups.

G. Urinalysis

No treatment-related change was noted at any dose level for the parameters assayed.

H. Sacrifice and pathology

1. Organ weights
Statistically significant decreases in mean absolute weights of lung (male only, $p < 0.05$) and liver (male only, $p < 0.05$), and statistically significant increases in mean relative organ weight ratios of brain (both sexes, $p < 0.01$), lung (females only, $p < 0.05$), liver (females only, $p < 0.01$), kidney (both sexes, $p < 0.01$) and testis (right side only, $p < 0.05$) were seen only in 20000 ppm groups.
Statistically significant decreases in mean body weight were seen throughout the study in the 20000 ppm males and females. Therefore, statistically significant decreased absolute weights and/or increased relative weights of various organs seen in the 20000 ppm group were attributed to the decreased body weights in these groups.

2. Gross and pathology
Macroscopic lesions were seen in the thymus (red patch/zone), teeth (deformity, malocclusion, overgrowing), stomach and small intestine (black content/zone), kidney (cyst), thyroid (dark red zone), skin (alopecia) and auricle (red/pale red zone). However, in these macroscopic incidences, there were no statistically significantly different as compared to the control.

3. Microscopic pathology
Histologically, test compound-related lesions were seen only in the kidneys of males in the 4000 and 20000 ppm groups and females of the 20000 ppm group. The kidney lesions, considered test compound related consisted of eosinophilic intranuclear inclusions in the proximal tubular epithelium in both males and females. In males, the inclusions were seen in 70% (7/10) and 100% (10/10) animals in the 4000 and 20000 ppm groups, respectively. There was also an increase in severity at the 4000 and 20000 ppm dosage levels. In females, slight to moderate inclusions were seen only in the 20000 ppm group at an incidence of 9/10 (90%).
These inclusions were most probably proteins since they were not stained with Feulgen, methyl green-pyronin, periodic acid-Schiff and Ziehl-Neelsen stain, but stained with hematoxylin-eosin stain. On electron-microscopic examination, the inclusions were of a granular-like structure with medium electron density. The ultrastructures of nucleus were normal, except for the inclusions. Other lesions were occasionally seen in the thymus (hemorrhage), lung (calcification of artery and epidermoid cyst), liver (microgranuloma and fat depletion), pancreas (inflammation), kidney (cyst and calcification), thyroid (hemorrhage) and auricle (chronic inflammation).
Similar kidney changes were observed in rats receiving propiverine hydrochloride (an anti-pollakiuria) at a gavage dose of 50 mg/kg/day for 52 weeks (Yamashita, K., Kuwata, M., Irimura, K., Morinaga, N.,

Kurokawa, K. and Ashizavva, M. (1990). J. Toxicol. Sci., 15, pp. 107-144), and these changes were not reported to be accompanied by any progressive or degenerative lesions of the cells. Thus, it was considered unlikely that the inclusions result in progressive change in a longer-term toxicity study of IM-0.

III. Conclusions

The effects of IM-0 on Crj: CD™ (SD) rats administered test compound through the diet for 13 weeks were decreased body weight gains, decreased food consumption values, increased serum alkaline phosphatase activity, and eosinophilic intranuclear inclusions in the proximal tubular epithelium of kidneys. The NOAEL was considered to be 800 ppm (48.9 mg/kg/day) in males, and 4000 ppm (275.9 mg/kg/day) in females.

BYI 02960-6CNA (6-chloronicotinic acid)

In vitro *genotoxicity - Bacterial assay for gene mutation*

Report:	**KIIA 5.8/18, [redacted] 1994 amended in 1997**
Title:	IC-0 – Reverse mutation study on bacteria
Report No & Document No MRID No:	G-942 M-195932-01-2 44988502 (submitted by Nippon Soda Co., Ltd., JPN)
Guidelines:	OECD 471 (1997), EPA Health Effects Test Guideline 84-2, JMAFF guideline 59 Nohsan No 4200
GLP	Yes (certified laboratory)

Executive Summary

The mutagenicity of IC-0, (6-chloronicotinic acid, Lot Nr.5, Purity 99.4%) was examined by the reverse mutation study using 4 strains (TA100, TA1535, TA98 TA1537) of *Salmonella typhimurium* and 1 strain (WP2 uvrA) of *Escherichia coli*. The study was performed with pre-incubation in both of the method with and without metabolic activation by S9 mix (Experimental No. 9854-1, 9854-2). IC-0 did not result in an increase in the number of reverse mutant colonies either in the presence or absence of metabolic activation.
Therefore, it is concluded that IC-0 is devoid of mutagenic activity under these experimental conditions.

I. Materials and Methods

A. Material

1. Test Material:	IC-0, 6-chloronicotinic acid
Description:	Crystal
Lot/Batch:	Nr.5
Purity:	99.4%
CAS:	5326-23-8
Stability of test compound:	5% DMSO solution stable for 4 hours at 24 °C

2. Control materials:

Negative: Culture medium
Solvent: DMSO
Positive: N-ethyl-N'-nitro-N-nitrosoguanidine for TA 1535 (at 5 µg/plate), TA 100 at 3 µg/plate and WP2 uvrA at 2 µg/plate 2-Nitrofluorene for TA 98 at 0.2 µg/plate, 9-Aminoacridine hydrochloride for TA 1537 at 80 µg/plate, 2-Aminoanthracene for the activating effect of the S9 mix in TA98 at 0.5 µg/plate, in TA 100 at 1 µg/plate, in TA 1535 and TA 1537 at 2 µg/plate and in WP2 uvrA at 10 µg/plate

3. Test organisms:

Species: *Salmonella typhimurium* LT2 mutants and Escherichia coli
Strain: Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102 and *Escherichia coli* WP2 uvrA strain
Source: Strains obtained from [redacted]

4. Test compound concentrations:

Experiment I and II: For all strains with or without S9 mix: 313, 625, 1250, 2500 and 5000 µg/plate

B. Study Design and methods

The experimental phase of the study was performed between October 22 to November 15, 1993 at [redacted]

The Salmonella/microsome test is a screening method which detects point mutations caused by chemical agents *in vitro*. Auxotrophic mutants of Salmonella typhimurium are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

Three test tubes and three minimum glucose agar plates were prepared for each test concentration and each of the solvent control and the positive controls. S9 mix was prepared from the liver of Sprague Dawley rats treated with 5,6-benzoflavone at 80 mg/kg for one day and phenobarbital at 30 - 60 mg/kg for 4 days intraperitoneally. The agar solution was complemented with 0.5 mM histidine and 0.5 mM biotin for *Salmonella typhimurium* and with 0.5 mM tryptophan for *Escherichia coli*.
In a preliminary test, no growth inhibition was observed at 2 500 mg/plate, the highest concentration tested. The highest concentration of the main experiment was set up at 5 000 µg/plate.
0.1mL of the test item solution, 0.5 mL of 0.1M sodium phosphate buffer or 0.5 mL of S9 mix for the metabolic activation method and 0.1 mL of the bacterial culture were put into a test tube and mixed. The mixture was incubated for 20 minutes at 37 °C under shaking. Then, 2 mL of the top agar was added to the tube, the mixture stirred and poured onto the agar plate. After the agar hardened, the plate was moved in the incubator and cultured at 37 °C.

The number of reverse mutant colonies was measured with the naked eyes, with the stereoscopic microscope or with the automatic colony counter. The presence of absence of the growth inhibition and the precipitate were observed with the stereoscopic microscope.

No statistical method was used. The test substance was characterized as positive in the test that fulfilled the following requirements: doubling of the spontaneous mutation rate of the solvent control, dose-response relationship, and reproducibility of the results.

II. Results and discussion

In the first main experiment, in the presence or absence of metabolic activation, no increase in the number of reverse mutant colonies was observed in any strain. Growth inhibition to all the *Salmonella* strains and precipitation of the test substance were observed at 5,000 µg/plate in presence of the metabolic activation. Growth inhibition to TA98 strain was observed without precipitation of the test substance at 5,000 µg/plate in absence of metabolic activation.
In the second main experiment, no increase in the number of reverse mutant colonies was observed in any strain in the presence or absence of the metabolic activation. Growth inhibition to TA98 strain was observed again without precipitation of the test substance at the highest concentration of 5,000 µg/plate in absence of metabolic activation.

Table 5.8-30: Mean numbers of revertant colonies (± SD) in the first experiment

Test item	Concentration (µg/plate)	TA100	TA1535	TA98	TA1537	WP2 uvrA
Without S9 mix						
Control	-	127 ± 2.65	10 ± 7.23	24 ± 4.36	11 ± 2.52	19 ± 2.65
Test item	313	116 ± 4.62	8 ± 2.65	26 ± 2.31	14 ± 3.21	17 ± 3.61
	625	109 ± 9.29	6 ± 1.15	26 ± 3.61	14 ± 5.03	22 ± 4.62
	1250	135 ± 4.51	8 ± 1.00	25 ± 2.31	19 ± 4.58	20 ± 5.77
	2500	122 ± 11.37	8 ± 2.52	30 ± 2.89	12 ± 4.04	24 ± 2.65
	5000	134 ± 8.72	4 ± 1.53	10 ± 5.00	10 ± 3.51	18 ± 3.21
ENNG	3	557 ± 25.01	-	-	-	-
ENNG	5	-	496 ± 65.77	-	-	-
2NF	0.2	-	-	88 ± 4.58	-	-
9AA	80	-	-	-	548 ± 26.76	-
ENNG	2	-	-	-	-	445 ± 37.87
With S9 mix						
Control	-	127 ± 4.51	14 ± 0.58	46 ± 6.56	20 ± 2.00	32 ± 12.06
Test item	313	121 ± 7.00	10 ± 4.16	47 ± 2.31	15 ± 2.65	36 ± 3.79
	625	113 ± 9.87	14 ± 2.00	42 ± 1.53	14 ± 3.61	28 ± 6.43
	1250	129 ± 14.00	11 ± 5.51	38 ±2.00	13 ± 1.53	31 ± 11.27
	2500	133 ± 9.24	22 ± 5.86	34 ± 7.00	20 ± 2.00	22 ± 4.73
	5000	90 ± 21.55	9 ± 2.00	34 ± 7.00	10 ± 4.04	28 ± 10.44
2AA	1	502 ± 23.39	-	13 ± 5.51	-	-
2AA	2	-	184 ± 9.17	-	164 ± 8.39	-
2AA	0.5	-	-	134 ± 4.36	-	-
2AA	10	-	-	-	-	663 ± 13.75

Table 5.8-31: Mean numbers of revertant colonies (± SD) in the second experiment

Test item	Concentration (µg/plate)	TA100	TA1535	TA98	TA1537	WP2 uvrA
Without S9 mix						
Control	-	127 ± 2.52	12 ± 3.06	24 ± 4.04	14 ± 3.21	24 ± 2.65
Test item	313	125 ± 16.04	9 ± 2.52	27 ± 4.04	11 ±2.00	22 ± 7.02
	625	132 ± 4.93	11 ± 2.31	35 ± 2.00	10 ±3.61	27 ± 6.35
	1250	138 ± 5.00	9 ± 6.08	25 ± 1.15	13 ± 5.00	26 ± 6.11
	2500	134 ± 4.36	15 ± 3.51	31 ± 8.72	14 ± 4.93	20 ± 4.04
	5000	134 ± 8.72	9 ± 2.52	18 ± 7.21	12 ± 1.73	23 ± 3.06
ENNG	3	657 ±25.00	-	-	-	-
ENNG	5	-	516 ± 24.99	-	-	-
2NF	0.2	-	-	91 ± 9.17	-	-
9AA	80	-	-	-	574 ± 40.04	-
ENNG	2	-	-	-	-	436 ± 9.07
With S9 mix						
Control	-	120 ± 6.08	15 ± 2.08	37 ± 2.52	12 ± 4.62	27 ± 5.20
Test item	313	124 ± 15.50	17 ± 4.51	43 ± 8.62	19 ± 2.31	24 ± 1.73
	625	107 ± 6.43	16 ± 5.20	45 ± 17.04	14 ± 3.79	21 ± 4.04
	1250	130 ± 9.61	11 ± 2.52	39 ± 8.74	14 ± 4.16	28 ± 6.00
	2500	114 ± 3.79	15 ± 3.21	39 ± 3.21	17 ± 2.52	23 ± 5.13
	5000	113 ± 11.02	22 ± 2.08	18 ± 1.15	18 ± 4.04	28 ± 4.58
2AA	1	652 ± 20.03	-	-	-	-
2AA	2	-	201 ± 17.67	-	148 ± 8.72	-
2AA	0.5	-	-	125 ± 14.57	-	-
2AA	10	-	-	-	-	682 ± 15.04

III. Conclusions

At 5,000 µg/plate, IC-0 inhibited the growth of TA98 strain in absence of metabolic activation and all the *Salmonella* strains in presence of the metabolic activation. IC-0 induced no increased in the number of reverse mutant colonies to any strains. In conclusion, IC-0 was negative under this experimental condition.

Acute oral toxicity

Report:	**KIIA 5.8/19, [redacted] 1993 amended in 1997**
Title:	IC-0, Acute oral toxicity in the rats
Report No & Document No MRID No:	G-0941 M-195930-01-2 44988420 (submitted by Nippon Soda Co., Ltd., JPN)
Guidelines:	OECD 401, EPA Health Effects Test Guideline 81-1, JMAFF guideline 59 Nohsan No 4200
GLP	Yes (certified laboratory)

Executive Summary

An acute oral toxicity study using 7 weeks old Sprague Dawley rats (5/sex/group) was performed with IC-0 (6-chloronicotinic acid, batch Nr.5, 99.4% of purity) administered via gavage at doses of 2000 and 5000 mg/kg. Clinical signs and mortality were observed for 14 days. Body weight was measured on days 0, 1, 2, 3, 7 and 14. A gross necropsy was performed on all animals that died during the observation period and on all animals that survived until the termination of the experiment.
No deaths were observed in any group of either sex. The acute oral LD50 values of IC-0 in rats is greater than 5,000 mg/kg for both sexes.
No signs of toxicity were observed in any groups of either sex. No unusual changes in body weight were observed in 2,000 mg/kg in males. Body weights in 5,000 mg/kg males decreased on day 1 to day 2 after the administration, and recovered from day 3. Body weights in 2,000 mg/kg females decreased on day 1 after the administration. Body weights in 5,000 mg/kg females decreased on days 1 to 3 after the administration and recovered thereafter. No abnormality was observed in any rats at necropsy.

I. Materials and Methods

A. Material

1. Test Material: IC-0, 6-chloronicotinic acid

Description:	Crystal
Lot/Batch:	Nr.5
Purity:	99.4%
CAS:	5326-23-8
Stability of test compound:	No analysis performed during the study

2. Vehicle and /or positive control: Tween 80-ion-exchange water

3. Test animals:

Species:	Rat
Strain:	Crj:CD (SD), SPF
Age:	7 weeks
Weight at dosing:	203.2 to 223.7 g in males and 144.1 to 154.1g in females
Source:	[redacted].
Acclimation period:	7 days
Diet:	Pelleted diet, MF® (Oriental Yeast Co., Ltd), *ad libitum*

Water:	Tap water, *ad libitum*	
Housing:	Animals were group caged conventionally in stainless steel mesh cages	
Environmental conditions:	Temperature:	22.4 ± 0.7 °C
	Humidity:	61.0 ± 2.9%
	Air changes:	Approximately 12 changes per hour
	Photoperiod:	Alternating 12-hour light and dark cycles.

B. Study Design and methods

1. In life dates

October 5 to 19, 1993 performed at [redacted]

2. Animal assignment and treatment

The substance was tested at 2000 and 5000 mg/kg in five male and five female Sprague Dawley rats per group. The animals were randomly allocated to cages. Following an overnight fast, the animals received a single dose of IC-0 (6-chloronicotinic acid, BYI02960-6-CNA) by gavage. The test substance was administered in Tween 80 - ion-exchange water at a volume of 10 mL/kg bw. Clinical signs and mortality rates were determined several times on the day of administration and subsequently at least once daily for an observation period of at least 14 days. Body weights were recorded prior to administration and on days 1, 2, 3, 7 and 14 and at death except on day 0. A gross necropsy was performed on all animals that died during the observation period and on all animals that survived until the termination of the experiment.

II, Results and discussion

A. Mortality

No deaths were observed in any groups of either sex. The acute oral LD50 values of IC-0 in rats were more than 5000 mg/kg in both sexes.

B. Clinical observations

No signs of toxicity were observed in any groups of either sex.

C. Body weight

No unusual changes in body weight were observed in 2,000 mg/kg in males. Body weights in 5,000 mg/kg males decreased on day 1 to day 2 after the administration, and recovered from day 3. Body weights in 2000 mg/kg females decreased on day 1 after the administration. Body weights in 5,000 mg/kg females decreased on days 1 to 3 after the administration and recovered thereafter.

D. Necropsy

No abnormality was observed in any rats at necropsy.

III. Conclusions

Under the experimental conditions of this study, the acute oral LD_{50} value of IC-0 in rats is greater than 5000 mg/kg for both sexes, meeting the criteria for EPA toxicity category IV.

Due to new guidance for TTC recommending to verify the genotoxic potential for metabolites above the threshold of 0.0025 µg/kg/day, two additional metabolites were tested in the Ames test and the in vitro micronucleus test (the metabolites were chosen as additional representatives to cover each metabolic paths detected with at least one representative metabolite):

- BYI 02960-acetic acid (representative for BYI 02960-glyoxylic acid, BYI 02960-AMCP-difluoroethanamine and BYI 02960-N-formyl/-N-acetyl-AMCP-difluoroethanamine)
- BYI 02960-amino-furanone (was the terminal metabolite after degradation of BYI 02960-difluoroethylaminofuranone)

BYI 02960-amino-furanone

In vitro *genotoxicity - Bacterial assay for gene mutation*

Report:	**KIIA 5.8/20, [redacted] A.; 2014**
Title:	BCS-AC74194 (BYI 02960-amino-furanone): *Salmonella typhimurium* reverse mutation assay
Report No & Document No	1626501 M-491203-01-1
Guidelines:	OECD 471 (1997); Commission Regulation (EC) No 440/2008 B13/14 (2008); EPA Health Effects Test Guideline (OPPTS 870.5100; 1998)
GLP	Yes (certified laboratory) except that no analytical analyses were performed during the study

Executive Summary

This study was performed to investigate the potential of BCS-AC74194 (BYI 02960-amino-furanone), a metabolite of BYI 02960 (batch N° SES 12288-26-8, 97.4% of purity) to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100. and TA 102. The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations: Pre-Experiment/Experiment I: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate; Experiment II: 33, 100, 333, 1000, 2500 and 5000 µg/plate. No precipitation of the test item occurred up to the highest investigated dose. The plates incubated with the test item showed normal background growth in all strains with or without S9 mix in both experiments up to 5000 µg/plate.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-AC74194 (BYI 02960-amino-furanone) at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, BCS-AC74194 (BYI 02960-amino-furanone) is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

I. Materials and Methods

A. Material

1. Test Material: BCS-AC74194 (BYI 02960-amino-furanone)
Description: xx
Lot/Batch: SES 12288-26-8
Purity: 97.4% (0.26% water)
CAS:
Stability of test compound: No analysis performed during the study

2. Control materials:
Negative: Culture medium
Solvent: DMSO
Positive: Sodium azide (Serva) for TA 1535 and TA 100 at 10 µg/plate,
4-Nitro-1,2-phenylene diamine (Sigma) for TA 1537 at 50 µg/plate and TA 98 at 10 µg/plate,
Methyl methane sulfonate (Sigma) for TA 102 at 2.0 µL/plate,
2-Aminoanthracene (Sigma Aldrich) for the activating effect of the S9 mix in all strains at 2.5 µg/plate for all strains except for TA 102 at 10.0 µg/plate

3. Test organisms:
Species: Salmonella typhimurium LT2 mutants
Strain: Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102
Source: Strains obtained from [redacted] Germany)

4. Test compound concentrations:

Experiment I:	First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Experiment II:	For TA 1535, TA 1537, TA98, TA100 and TA 102 with or without S9 mix: 33, 100, 333, 1000, 2500 and 5000 µg/tube

B. Study Design and methods

The experimental phase of the study was performed between 27 May 2014 and 13 June 2014 at [redacted] Germany.

The Salmonella/microsome test is a screening method which detects point mutations caused by chemical agents *in vitro*. Auxotrophic mutants of Salmonella typhimurium are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

1. Plate incorporation assay (experiment I)

DMSO (0.1 mL) containing BCS-AC74194 or controls were added to glass vessels with 0.1 mL of bacterial cultures grown overnight, 0.5 mL of S9 mix or buffer and 2 mL of soft agar. The mixture was placed in a waterbath at 45 °C for 30 seconds, shaken and overlaid onto Petri dishes containing solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were scored using the Petri Viewer Mk2. Three plates were used, both with and without S9 mix, for each strain and dose. The doses for the first trial were routinely determined on the basis of a standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses.

2. Pre-incubation assay (experiment II)

An independent repeat was performed as pre-incubation of the previously described mixture in a water bath at 37 °C for 60 minutes. At the end of the preincubation period 2 mL of molten soft agar were added to the tubes, the content mixed and plated onto Petri dishes with solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were also scored using an automated colony counter.

3. Assessment criteria

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100 and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed. A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration. An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and discussion

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used. No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with or without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-AC74194 (BYI 02960-amino-furanone) at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.
Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

It can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

III. Conclusions

No indication of mutagenic effects of BCS-AC74194 (BYI 02960-amino-furanone), a metabolite of BYI 02960, could be found at assessable doses of up to 5000 µg/plate in any of the *Salmonella typhimurium* strains used in the assay.

In vitro *genotoxicity (somatic cells) - Bone marrow or micronucleus*

Report:	**KIIA 5.8/21, [redacted]: 2014**
Title:	BCS-AC74194 (BYI 02960-amino-furanone): *In vitro* Micronucleus Test in Human lymphocytes
Report No & Document No	1626502 M-497695-01-1
Guidelines:	OECD 487 (2010); Commission regulation (EU) No 640-2012 B49 (2012)
GLP	Yes (certified laboratory)

Executive Summary

The test item BCS-AC74194 (BYI 02960-amino-furanone) was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in two independent experiments according to the following design:

	Without S9 mix		**With S9 mix**
	Experiment I	**Experiment II**	**Experiment I & II**
Exposure period (hrs)	4	20	4
Recovery (hrs)	16	-	16
Cytochalasin B exposure (hrs)	20	20	20

Preparation interval (hrs)	40	40	40
Total culture period (hrs)*	88	88	88

*exposure started 48 hours after culture initiation

In each experimental group two parallel cultures were analysed. Within each culture 1000 binucleated cells were evaluated for cytogenetic damage. The highest applied concentration in this study (1000.0 µg/mL of the test item, approx. 10 mM) was chosen with regard to the molecular weight of the test item and with respect to the current OECD Guideline 487. Dose selection in the cytogenetic experiment was performed considering the toxicity data in accordance with OECD Guideline 487. In both independent experiments, no relevant cytotoxicity was observed up to the highest applied concentration. In the absence and the presence of S9 mix, no increase in the number of micronucleated cells was observed after treatment with BCS-AC74194 (BYI 02960-amino-furanone). Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

Under the experimental conditions reported BCS-AC74194 (BYI 02960-amino-furanone) did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes. Therefore, BCS-AC74194 (BYI 02960-amino-furanone) is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required or precipitating concentration.

I. Materials and Methods

A. Material

1. Test Material: BCS-AC74194 (BYI 02960-amino-furanone)
Description: XX
Lot/Batch: SES 12288-26-8
Purity: 97.4% (0.26% water)
CAS: not available
Stability of test compound: No analysis performed during the study

2. Control materials: Negative: Culture medium with 10% deionized water
Positive: Without S9 mix: mitomycin C (pulse treatment); 3.0 ug/mL, Demecolcin (continuous treatment); 75 ng/mL
With S9 mix: cyclophosphamide; 15 ug/mL in Experiment I, 12.5 ug/mL in Experiment II

3. Test system:
Species: Human female donors
Cell type: Peripheral blood lymphocytes
Culture conditions: Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hrs after blood collection.

4. Test compound concentrations:

Dose selection/Experiment I:	Ten concentrations ranging from 6.5 to 1000 ug/mL (with and
without precipitation	without the S9 mix) were assessed for cytotoxicity and
Experiment II:	1000 ug/mL

B. Test performance

The experimental phase of the study was performed from 04 July 2014 to 16 July 2014 at [redacted], Germany.

1. Cytogenetic Experiments

Pulse exposure

About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hrs the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G". Washed cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until slide preparation.

Continuous exposure (without S9 mix)

About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". Washed cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until slide preparation.

2. Preparation of slides

The cultures were harvested by centrifugation 40 hrs after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

3. Acceptance criteria

The study was considered valid as the following criteria were met: micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area

of the main nucleus. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the cytokinesis-block proliferation index (CBPI) was determined in 500 cells per culture and cytotoxicity was expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) was equivalent to 100% cytostasis**.**

4. Evaluation of the results

Many experiments with human lymphocytes have established a range of micronucleus frequencies acceptable for control cultures in normal volunteer donors. The current historical data range together with the statistical significance, confirmed by the Chi-square test ($\alpha = 0.05$), should be considered for classification of the test item. An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

II. Results and discussion

The test item BCS-AC74194 (BYI 02960-amino-furanone), dissolved in deionised water, was assessed in two independent experiments for its potential to induce micronuclei in human lymphocytes in vitro in the absence and presence of metabolic activation by S9 mix. Precipitation of the test item in the culture medium was observed microscopically at the end of treatment in Experiment II in the absence of S9 mix at 571.4 µg/mL and above. No relevant influence on osmolarity and pH was observed. No relevant cytotoxicity, indicated by reduced CBPI and described as cytostasis could be observed up to the highest applied concentration.

In both independent experiments, in the absence and presence of S9 mix, no biologically-relevant increase in the number of cells carrying micronuclei was observed. The micronucleus rates of the cells after treatment with BCS-AC74194 (BYI 02960-amino-furanone) (0.20 – 0.50 % micronucleated cells, were within the range of the solvent control values (0.20 – 0.70% micronucleated cells) and clearly within the range of the laboratory historical control data. In both experiments, either Demecolcin (75.0 ng/mL), MMC (3.0 µg/mL) or CPA (12.5 or 15.0 µg/mL) were used as positive controls and showed expected increases in cells with micronuclei.

III. Conclusions

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes. Therefore, BCS-AC74194 (BYI 02960-amino-furanone) is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required or precipitating concentration.

BYI 02960-acetic acid (BCS-CQ74373)

In vitro *genotoxicity - Bacterial assay for gene mutation*

Report:	**KIIA 5.8/22, [redacted] A.; 2013**
Title:	BYI 02960-acetic acid (BCS-CQ74373): *Salmonella typhimurium* reverse mutation assay
Report No & Document No	1558101 M-463046-01-1

Guidelines:	OECD 471 (1997); Commission Regulation (EC) No 440/2008 B13/14 (2008); EPA Health Effects Test Guideline (OPPTS 870.5100; 1998)
GLP	Yes (certified laboratory) except that no analytical analyses were performed during the study

Executive Summary

This study was performed to investigate the potential of BYI 02960-acetic acid (BCS-CQ74373) to induce gene mutations according to the plate incorporation test (experiment I) and the preincubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations: Pre-Experiment/Experiment I: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate; Experiment II: 33, 100, 333, 1000, 2500 and 5000 µg/plate. No precipitation of the test item occurred up to the highest-investigated dose. The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used. No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation in both experiments.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BYI 02960-acetic acid (BCS-CQ74373) at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally-acknowledged border of biological relevance. Appropriate reference mutagens were used as positive controls and showed an expected increase of induced revertant colonies.

It can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, BYI 02960-acetic acid (BCS-CQ74373) is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960-acetic acid (BCS-CQ74373)
Description: not indicated
Lot/Batch: MXM 7075-3-1
Purity: 98.9% w/w
CAS: not available
Stability of test compound: No analysis performed during the study

2. Control materials:
Negative: Culture medium
Solvent: DMSO
Positive: Sodium azide (Serva) for TA 1535 and TA 100 at 10 µg/plate,

4-Nitro-1,2-phenylene diamine (Sigma) for TA 1537 at 50 µg/plate and TA 98 at 10 µg/plate,
Methyl methane sulfonate (Sigma) for TA 102 at 2.0 µL/plate,
2-Aminoanthracene (Sigma Aldrich) for the activating effect of the S9 mix in all strains at 2.5 µg/plate for all strains except for TA 102 at 10.0 µg/plate

3. Test organisms:

Species:	*Salmonella typhimurium* LT2 mutants
Strain:	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102
Source:	Strains obtained from [redacted] Germany)

4. Test compound concentrations:

Experiment I:	First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Experiment II:	For TA 1535, TA 1537, TA98, TA100 and TA 102 with or without S9 mix: 33, 100, 333, 1000, 2500 and 5000 µg/tube

B. Study Design and methods

The experimental phase of the study was performed between 19 June 2013 and 08 July 2014 [redacted] Germany.

The *Salmonella*/microsome test is a screening method which detects point mutations caused by chemical agents *in vitro*. Auxotrophic mutants of *Salmonella typhimurium* are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

1. Plate incorporation assay (experiment I)

DMSO (0.1 mL) containing BCS-AC74194 or controls were added to glass vessels with 0.1 mL of bacterial cultures grown overnight, 0.5 mL of S9 mix or buffer and 2 mL of soft agar. The mixture was placed in a waterbath at 45 °C for 30 seconds, shaken and overlaid onto Petri dishes containing solid agar. After 48 hours of incubation at 37 °C, the numbers of revertant colonies were scored using the Petri Viewer Mk2. Three plates were used, both with and without S9 mix, for each strain and dose. The doses for the first trial were routinely determined on the basis of a standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses.

2. Pre-incubation assay (experiment II)

An independent repeat was performed as pre-incubation of the previously described mixture in a water bath at 37 °C for 60 minutes. At the end of the preincubation period, 2 mL of molten soft agar were added to the tubes, the content mixed and plated onto Petri dishes with solid agar. After 48 hours of

incubation at 37 °C, the numbers of revertant colonies were also scored using an automated colony counter.

3. Assessment criteria

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100 and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed. A dose-dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration. An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment. A dose-dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and discussion

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used. No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with or without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BYI 02960-acetic acid (BCS-CQ74373) at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance. Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

It can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

III. Conclusions

No indication of mutagenic effects of BYI 02960-acetic acid (BCS-CQ74373), a metabolite of BYI 02960, could be found at assessable doses of up to 5000 µg/plate in any of the *Salmonella typhimurium* strains used in the assay.

In vitro *genotoxicity (somatic cells) - Bone marrow or micronucleus*

Report:	**KIIA 5.8/23, [redacted]; 2013**
Title	BYI 02960-acetic acid (BCS-CQ74373): Micronucleus Test in Human Lymphocytes *in vitro*
Report No & Document No	1558102 M-472828-01-1

Guidelines:	OECD 487 (2010); Commission regulation (EU) No 640-2012 B49 (2012)
GLP	Yes (certified laboratory)

Executive Summary

The test item BYI 02960-acetic acid (BCS-CQ74373), dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes in vitro in two independent experiments.
The following study design was performed:

	Without S9 mix		With S9 mix
	Experiment I	**Experiment II**	**Experiment I & II**
Exposure period (hrs)	4	20	4
Recovery (hrs)	16	-	16
Cytochalasin B exposure (hrs)	20	20	20
Preparation interval (hrs)	40	40	40
Total culture period (hrs)*	88	88	88

*exposure started 48 hours after culture initiation

In each experimental group two parallel cultures were analysed. Within each culture 1000 binucleated cells were evaluated for cytogenetic damage. The highest applied concentration in this study (2647.0 µg/mL of the test item, approx. 10 mM) was chosen with regard to the molecular weight of the test item and with respect to the current OECD Guideline 487. Dose selection in the cytogenetic experiment was performed considering the toxicity data in accordance with OECD Guideline 487. In both independent experiments, no relevant cytotoxicity was observed up to the highest applied concentration.

In the absence and the presence of S9 mix, no increase in the number of micronucleated cells was observed after treatment with the test item.. However, in Experiment II in the presence of S9 mix, one single statistically significant increase in micronucleated cells was observed after treatment with 1512.6 ug/mL (1.50 %). Since the value is within the range of the historical control data (0.20 – 1.65 % micronucleated cells for organic solvents), this finding was regarded as biologically irrelevant. Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

Under the experimental conditions reported, the test item did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes. Therefore, BYI 02960-acetic acid (BCS-CQ74373) is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

I. Materials and Methods

A. Material

1. Test Material: BYI 02960-acetic acid (BCS-CQ74373)
Description: not indicated
Lot/Batch: MXM 7075-3-1
Purity: 98.9% w/w
CAS:
Stability of test compound: No analysis performed during the study

2. Control materials: Negative: Culture medium with 0.5% DMSO
Positive: Without S9 mix: mitomycin C (pulse treatment); 2.0 ug/mL,
Demecolcin (continuous treatment); 125 ng/mL
With S9 mix: cyclophosphamide; 17.5 ug/mL in Experiment I, 12.5 ug/mL in Experiment II

3. Test system:
Species: Human female donor
Cell type: Peripheral blood lymphocytes
Culture conditions: Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hrs after blood collection.

4. Test compound concentrations:
Dose selection/Experiment I: Ten concentrations ranging from 17.2 to 2647.0 ug/mL (with and without the S9 mix) were assessed for cytotoxicity and without precipitation
Experiment II: 2647.0 ug/mL

B. Test performance

The experimental phase of the study was performed from 03 July 2013 to 27 August 2013 [redacted] Germany

1. Cytogenetic Experiments

Pulse exposure

About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hrs the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G". Washed cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until slide preparation.

Continuous exposure (without S9 mix)

About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation

for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". Washed cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until slide preparation.

2. Preparation of slides

The cultures were harvested by centrifugation 40 hrs after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

3. Acceptance criteria

The study was considered valid as the following criteria were met: micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the cytokinesis-block proliferation index (CBPI) was determined in 500 cells per culture and cytotoxicity was expressed as % cytostasis. A CBPI of 1 (i.e., all cells are mononucleate) was equivalent to 100% cytostasis.

4. Evaluation of the results

Many experiments with human lymphocytes have established a range of micronucleus frequencies acceptable for control cultures in normal volunteer donors. The current historical data range together with the statistical significance, confirmed by the Chi square test ($\alpha < 0.05$), should be considered for classification of the test item. An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

II. Results and discussion

The test item BYI 02960-acetic acid (BCS-CQ74373), dissolved in DMSO, was assessed in two independent experiments for its potential to induce micronuclei in human lymphocytes in vitro in the absence and presence of metabolic activation by an S9 mix. The highest treatment concentration in this study, 2647.0 µg/mL (approx. 10 mM) was chosen with regard to the molecular weight of the test item and with respect to the OECD Guideline 487 for the in vitro mammalian cell micronucleus test. No precipitation of the test item in the culture medium was observed. No relevant influence on osmolarity was observed. No relevant cytotoxicity, indicated by reduced CBPI and described as cytostasis could be observed up to the highest applied concentration.

In both independent experiments, in the absence and presence of S9 mix, no biologically-relevant increase in the number of cells carrying micronuclei was observed. However, in Experiment II in the

presence of S9 mix one single statistically significant increase in micronucleated cells was observed after treatment with 1512.6 Jg/mL (1.50 %). Since the value is within the range of the historical control data (0.20 – 1.65 % micronucleated cells for organic solvents), the finding was regarded as biologically irrelevant.

In both experiments, either Demecolcin (125.0 ng/mL), MMC (2.0 µg/mL) or CPA (12.5 or 17.5 µg/mL) were used as positive controls and showed expected increases in cells with micronuclei.

III. Conclusions

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes. Therefore, BYI 02960-acetic acid (BCS-CQ74373) is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

KIIA 5.9 - Medical and clinical data

KIIA 5.9.1 - Report on medical surveillance on manufacturing plant personnel

The active ingredient has been produced in 2 campaigns in 2008 and 2010. Routine occupational medical examinations of the workers involved in production, not directly correlated to the campaigns, showed no abnormalities.

KIIA 5.9.2 - Report on clinical cases and poisoning incidents

As the active ingredient has not yet been marketed, no such experiences exist.

KIIA 5.9.3 - Observations on general population exposure & epidemiological studies

As the active ingredient has not yet been marketed, no such experiences exist.

KIIA 5.9.4 - Clinical signs and symptoms of poisoning and details of clinical test

As no poisonings in human have occurred, no information is available.

KIIA 5.9.5 - First aid measures

- Remove patient from exposure terminate exposure under self-protection
- Thorough skin decontamination with copious amounts water and soap, if available with polyethylenglykol 300 followed by water
  <u>*Note:*</u> Most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polythyleneglykol 300 is not required
- Flushing of the eyes with lukewarm water for 15 minutes
- Induction of vomiting should only be considered if a significant amount has been swallowed (more than a mouthful), 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
- <u>*Note*:</u> Induction of vomiting is forbidden, if a formulation containing organic solvents has been ingested!

KIIA 5.9.6 - Therapeutic regimes

- Gastric lavage does not seem to be required in regard of the low toxicity
- The application of activated charcoal and sodium sulphate may be considered in significant ingestions
- As there is no antidote, treatment has to be symptomatic and supportive.

KIIA 5.9.7 - Expected effects & duration of poisoning as a function of exposure

As no poisonings in human have occurred, no information is available.

KIIA 5.9.8 - Effects & duration of poisoning as a function of time

As no poisonings in human have occurred, no information is available.

KIIA 5.9.9 - Dermal penetration

An *in vivo* dermal penetration study with the plant protection product has been conducted and is reported in Annex IIIAI point 7.6.1 and a comparative *in vitro* dermal absorption study using human

and rat skin has been conducted with the plant protection product and is reported in Annex IIIAI point 7.6.2.

KIIA 5.10 - Other/special studies

Report:	**KIIA 5.10/01, [redacted] M., 2010**
Title:	BYI 02960, Biokinetic in the plasma of rats following 7 days exposure through the diet
Report No & Document No	SA 09334 M-385777-01-2
Guidelines:	Non applicable
GLP	Not a GLP study but GLP certified laboratory

Executive Summary

The plasma concentration of BYI 02960, (batch number 2009-000239 beige powder, 96.2% (w/w) purity), was assessed at three time points after continuous dietary administration for seven days. Groups of 5 male and 5 female Wistar rats received the diet admixed with the test substance at a concentration of 400 ppm (equating approximately to 22.6 mg/kg body weight/day in males and 32.4 mg/kg body weight/day in females).

During the first 15 days of acclimatization, the daily light/dark rhythm was 7 a.m. - 7 p.m. Rats being known to preferably eat during the dark period, the daily light/dark rhythm of rats was changed from November 25, 2009 (pre-study Day 16, *i.e.* 14 days prior to first day of treatment) onwards, *i.e.* dark period was from 2 a.m. to 2 p.m. and light period was from 2 p.m. to 2 a.m., in order to allow the blood sampling during the working day. Clinical signs were observed daily. Body weights were measured just prior to the start of admixed food administration (Study Day 1) and on Study Day 8. Food consumption was recorded on Study Days 1 - 4, 4 - 7 and 7 - 8.

Whole blood was collected on heparin for plasma preparation from treated animals at three time points on Study Day 8 (8 a.m., 2 p.m. and 5 p.m.). The day after the end of the treatment period, animals were sacrificed without necropsy.

Dietary administration of BYI 02960 induced no mortalities or treatment-related clinical signs.

The results obtained indicated a small sex difference in the plasma concentrations of BYI 02960, values being slightly higher for females than for males.

In male rats, a Cmax of 8.3 mg/L was measured at the time of collection of 8 a.m. In female rats, a Cmax of 9.4 mg/L was measured at the time collection of 2 p.m. However, in view of the inter-individual variability, the concentrations of BYI 02960 in plasma were considered similar between the three times of blood collection for both sexes (between 7.8 and 8.3 mg/L for males and between 8.8 and 9.4 mg/L for females).

According to these results and using the experimental study design, blood samples collected between 8 a.m. and 5 p.m. are adequate for measuring BYI 02960 concentrations in plasma around the Cmax for both sexes, which correspond to a Tmax at between 6 hours and 15 hours after the light switch off for both sexes.

In conclusion, in the environmental conditions of the long-term studies (light: 7 a.m. to 7 p.m. & dark period: 7 p.m. to 7 a.m.) and assuming that animals are starting eating food when the light is switched off, the blood collection can be performed between 1 a.m. and 10 a.m. in order to measure the maximum concentration of BYI 02960.

I. Materials and Methods

A. Material

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable at 45.5 and 5000 ppm for at least 100 days
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Rat
Strain:	Rj:WI (IOPS HAN)
Age:	16 weeks
Weight at dosing:	467 to 516 g for males and 290 to 302 g for females
Source:	[redacted] France
Acclimation period:	28 days
Diet:	Powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Rats were housed individually in suspended, stainless steel, wire-mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C
	Humidity: 55 ± 15%
	Air changes: Approximately 12 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (During the first 15 days of acclimation 7 a.m. - 7 p.m., 2 a.m. - 2 p.m. thereafter)

B. Study design

1. In life dates

November 10 to December 15, 2009 performed at [redacted], France.

2. Animal assignment and treatment

Fourteen (7 males and 7 females) Wistar Rj:WI (IOPS HAN) rats were obtained from [redacted] France.

They were acclimatized to laboratory conditions for twenty-eight days prior to the treatment and were approximately 16 weeks old at the start of treatment. All animals were weighed at least weekly and checked daily for clinical signs, moribundity and mortality. The food consumption was measured during the Week 4 of the acclimatization phase to check that animals were well acclimatized to their

housing conditions. At the time of randomization, all animals were weighed. An automatic procedure (XMS Path/Tox Version 4.2.2) 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. Ten rats (five males, five females) were selected for the study. They were within ± 20% of the mean body weight on the day of randomization.
The dose level selected is the intermediate dose level of the chronic/carcinogenicity study in the rat with the test substance and was chosen to support effects seen at 400 ppm in that study. Groups of 5 male and 5 female rats were given the appropriate admixed diet.

3. Diet preparation and analysis
The test substance was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required dietary concentration. A small amount of acetone solution was used to facilitate test substance mixture in the diet (acetone evaporated during the mixture process). There was one diet preparation. When not in use, the diet formulation was stored at room temperature. The stability of the test substance in the diet was demonstrated in a previous study (SA 09014) at concentrations of 455 and 5000 ppm for at least 100 days at room temperature.

The homogeneity of the test substance in the diet was verified to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. The homogeneity and concentration results ranged between 93 and 95% of the nominal concentration and were therefore within the in-house target ranges.

C. Methods

1. Daily observations
All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once each day starting on Study Day 1 and every day throughout the study.

2. Body weight
Each animal was weighed just prior to the start of admixed food administration (Study Day 1) and on Study Day 8.

3. Food consumption
The weight of food supplied and of that remaining at the end of the food consumption period was recorded on Study Days 1 - 4, 4 - 7 and 7 - 8 for all animals during the treatment period. Food spillage was also noted.

The weekly mean achieved dosage intake in mg/kg body weight/day for Week 1 was calculated.

4. Blood sampling and analysis
Whole blood was collected from treated animals at three time points on Study Day 8 (8 a.m., 2 p.m. and 5 p.m.) following 7 days of exposure. These three time points corresponded to 6 hours after the light switch-off, 12 hours and 15 hours, respectively. Animals were assumed starting eating when the light

was switched off. Blood collection was performed on stock animals for validation of the analytical method.
These samples were taken by puncture of the sublingual vein, into heparinised vials. Prior to blood sampling animals were anesthetized using Isoflurane. Plasma was then prepared by centrifugation. The plasma samples were placed on ice and sent to the Analytical Department for determination of the concentration of the test substance. All samples awaiting processing were stored in the dark at approximately -70 °C until analysis.

5. Necropsy
At Study Day 9, animals were euthanized by inhalation of carbon dioxide and discarded without necropsy or were used for training purposes.

II. Results and discussion

A. In Life observations

There were no mortalities and no treatment-related clinical signs observed during the study. The few clinical signs observed (hair loss observed on two females) were considered not to be related to BYI 02960 administration. Body weight evolution and food consumption were similar to the one observed in the chronic and carcinogenicity study.

B. Achieved dosages

The mean achieved dose levels, expressed in mg/kg body weight/day, received by the animals during the study were as follows:

Table 5.10-1: Achieved dosages

Mean achieved dietary intake of BYI 02960 (Week 1)		
Diet concentration ppm	**Male mg/kg/day**	**Female mg/kg/day**
400	22.6	32.4

C. Biokenetic results

The individual and mean concentrations of BYI 02960 in plasma samples were used to identify the appropriate maximal concentration (Cmax) and the corresponding time point of collection for the long-term studies.
The results obtained indicated a small sex difference in the plasma concentrations of BYI 02960, values being slightly higher for females than for males.
In male rats, a Cmax of 8.3 mg/L was measured at the time of collection of 8 a.m. In female rats, a Cmax of 9.4 mg/L was measured at the time collection of 2 p.m. However, in view of the inter-individual variability, the concentrations of BYI 02960 in plasma were considered similar between the three times of blood collection for both sexes (between 7.8 and 8.3 mg/L for males and between 8.8 and 9.4 mg/L for females).

Table 5.10-2: Plasma concentration of BYI 02960 in male

Animal reference	Time collection	Plasma concentration of BYI 02960		
		Individual data (mg/L)	Mean (mg/L)	SD
TT1M4982	8 a.m.	6.14	8.3	1.3
TT1M4983		8.63		
TT1M4984		8.59		
TT1M4985		8.56		
TT1M4986		9.77		
TT1M4982	2 p.m.	5.87	7.8	1.3
TT1M4983		7.14		
TT1M4984		8.73		
TT1M4985		8.36		
TT1M4986		8.90		
TT1M4982	5 p.m.	4.73	8.0	2.0
TT1M4983		9.98		
TT1M4984		8.30		
TT1M4985		7.77		
TT1M4986		9.05		

Table 5.10-3: Plasma concentration of BYI 02960 in female

Animal reference	Time collection	Plasma concentration of BYI 02960		
		Individual data (mg/L)	Mean (mg/L)	SD
TT1F4987	8 a.m.	7.76	9.0	1.3
TT1F4988		9.33		
TT1F4989		8.66		
TT1F4990		11.0		
TT1F4991		8.28		
TT1F4987	2 p.m.	8.16	9.4	1.2
TT1F4988		9.86		
TT1F4989		9.13		
TT1F4990		11.1		
TT1F4991		8.56		
TT1F4987	5 p.m.	7.56	8.8	1.6
TT1F4988		9.85		
TT1F4989		7.91		
TT1F4990		11.2		
TT1F4991		7.67		

Therefore, according to these results and using the experimental study design, blood samples collected between 8 a.m. and 5 p.m. are adequate for measuring BYI 02960 concentrations in plasma around the C_{max} for both sexes.

III. Conclusions

In conclusion, in the environmental conditions of the long-term studies (light: 7 a.m. to 7 p.m. & dark period: 7 p.m. to 7 a.m.) and assuming that animals are starting eating food when the light is switched off, the blood collection can be performed between 1 a.m. and 10 a.m. in order to measure the maximum concentration of BYI 02960.

Report:	**KIIA 5.10/02, [redacted] M.; 2011**
Title:	BYI 02960, 28-day immunotoxicity study in female Wistar rats by dietary administration
Report No & Document No	SA 10353 M-414754-01-2
Guidelines:	U.S.E.P.A., OPPTS Series 870, Health Effects Testing Guidelines, N° 870.7800 (August 1998)
GLP	Yes certified laboratory

Executive Summary

BYI 02960 (batch number 2009-000239: a beige powder, 96.2% w/w purity), was administered continuously via dietary administration to separate groups of female Wistar rats (10/group) at concentrations of 125, 600 and 3000 ppm (equating approximately to 10, 50, 230 mg/kg body weight/day) for at least 28 days. A similarly constituted group received untreated diet and acted as a control group. An additional group of 10 female rats were administered cyclophosphamide (immunosuppressive agent) daily by gavage for at least 28 days at concentration of 3.5 mg/kg body weight/day and acted as positive control group. Four days before necropsy, all animals were immunized with Sheep Red Blood Cell (SRBC) antigen by intravenous injection of 2.5 x 108 SRBC/animal via the tail vein. Animals were observed daily for mortality and clinical signs. Body weight and food consumption were recorded once weekly. A detailed physical examination was performed once during the acclimatization phase and at least weekly throughout the study. Blood samples were collected from the retro-orbital venous plexus of each surviving animal on Study Day 30 (just before necropsy) for specific anti-SRBC immunoglobulin M (IgM) analysis. All animals were necropsied, gross pathology observations were performed and selected organs (spleen and thymus) weighed.

Dietary administration of BYI 02960 for at least 28 days to female Wistar rats at dose levels of 125, 600 and 3000 ppm induced no mortalities or treatment-related clinical signs.

At 3000 ppm, mean body weight was reduced by between 6 and 11% from Study Day 8 to 29 when compared to the control group. The effect was statistically significant on the intervals Day 1 to 8 and Day 8 to 15. There was no body weight gain/day between Study Day 1 and 8. Between Study Day 1 and 29, the cumulative body weight was reduced by 23%, when compared to the control group.
Mean food consumption was reduced by approximately 34% on Study Day 8 and by between 9 and 13% onwards, when compared to control group although not statistically significant. A lower mean terminal body weight was observed in treated females when compared to the controls (- 6%, not statistically significant).
For the immunological response, the results obtained in the control group after immunization with the antigen SRBC and those obtained with the positive control confirmed the ability of the system to detect the immuno-suppressive effects and confirmed the validity of the study design.
No relevant change was noted in anti-SRBC IgM concentrations up to 3000 ppm, compared to controls.

Taken into account all systemic effects of BYI 02960, the No Observed Adverse Effect Level (NOAEL) following continuous dietary administration to female Wistar rats for at least 28 days was 600 ppm (equating approximately to 50 mg/kg/day).

In conclusion, BYI 02960 was considered not to have any immunotoxic potential in female Wistar rats when given in the diet at dose levels up to 3000 ppm (corresponding to approximately 230 mg/kg/day) for at least 28 days.

I. MATERIALS AND METHODS

A. Materials:

1. Test Material:	BYI 02960
Description:	Beige powder
Lot/Batch:	2009-000239
Purity:	96.2%
CAS:	951659-40-8
Stability of test compound:	Stable at 70 and 2500 ppm for at least 110 days at room temperature
2. Vehicle and /or positive control:	Cyclophosphamide
Description:	white powder
Lot/Batch:	068K1131
Purity:	98%
CAS:	6055-19-2
Stability of test compound:	Stable at 1and 3g/L at +5 °C (± 3 °C)
3. Test animals:	
Species:	Rat
Strain:	Rj:WI (IOPS HAN)
Age:	7 weeks
Weight at dosing:	165 to 206 g for females
Source:	[redacted] France.
Acclimation period:	12 days
Diet:	Powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) ad libitum
Water:	Filtered and softened tap water, ad libitum
Housing:	Rats were housed individually in suspended, stainless steel, wire-mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C Humidity55: 55 ± 15% Air changes: Approximately 12 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study design:

1. In life dates

March 23 to May 03, 2011 performed at [redacted], France.

2. Animal assignment and treatment

Fifty-five female Wistar Rj:WI (IOPS HAN) rats were obtained from [redacted] France.

They were acclimatized to laboratory conditions for twelve days prior to the treatment and were approximately 7 weeks old at the start of treatment. All animals were weighed at least weekly and checked daily for clinical signs, moribundity and mortality. At the time of randomization, all animals were weighed. An automatic procedure (XMS PathTox Version 4.2.2) 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. Fifty female rats were selected for the study. Selected animals were in a weight range from 165 to 206 g for the females on the start of treatment.

The dose levels of 0, 125, 600 and 3000 ppm were set after evaluation of the general systemic toxicities seen in previous studies conducted with this substance.

Table 5.10-4: Study design

Group	Test Substance	Dose level (ppm)	Number of animals Per group
		Female	
1	Control	0	10
2	BYI 02960	125	10
3		600	10
4		3000	10
Group	Positive control	Dose level (mg/kg/day)	Number of animals per group
5	Cyclophosphamide	3.5	10

All groups treated by the test substance received the appropriate dietary concentrations at a constant dose level. Control group and the group treated by the immunosuppressive agent cyclophosphamide received untreated diet.

Rats received the cyclophosphamide formulation by gavage (3.5 mg/kg bw/day) at a dosage volume of 5 mL/kg body weight. The volume administered to each rat was adjusted on the most recently recorded body weight.

3. Diet preparation and analysis of the test substance

The test substance in acetone solution was incorporated into the diet to provide the required dietary concentrations. For control group, a control formulation was prepared by adding an equivalent volume of acetone into the diet. There was one preparation for each concentration. When not in use, the diet formulations were stored at room temperature.

The stability of the dietary formulation was determined in a previous study (SA 08337) at 70 and 2500 ppm over a 110 days period of storage at room temperature. Additionally, the stability of

BYI 02960 was checked at 3000 ppm for a time period that covers the period of storage and usage for the study. A single diet sample of BYI 02960 at 3000 ppm was taken and analyzed after having been kept at room temperature for 34 days.

The homogeneity of test substance in diet was verified during the study for the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. Dietary levels of the test substance were verified for each concentration.
The homogeneity and concentration results ranged between 90 and 96% of the nominal concentration and were therefore within in-house target of 85 and 115% for a dietary mix.
In addition, BYI 02960 was found stable at 3000 ppm in rodent diet over a storage period of 34 days at room temperature.

4. Diet preparation and analysis of the positive control substance (cyclophosphamide)
The dosing formulation of cyclophosphamide was prepared by suspending the substance in sterilized water to produce the required dosing concentration and stored in air-tight light resistant containers at approximately +5 (± 3 °C) when not in use. There were two preparations during the study and, due to an insufficient volume of the second formulation (F2) 23 mL were taken from the second formulation (F2) (batch number 120M1253: a white powder, 100.6% purity).
The homogeneity of cyclophosphamide in vehicle was verified on the first formulation to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. Concentration of the positive control substance in vehicle was verified for each preparation.
The stability of cyclophosphamide in vehicle has been demonstrated in previous studies at concentrations of 0.1, 1 and 3 g/L for a time period which covers the period of storage and usage for the current study.

4. Statistics
The following variables were analyzed: body weight parameters, body weight change parameters calculated according to time intervals, average food consumption/day parameters calculated according to time intervals, terminal body weight, absolute and relative organ weights parameters, immunological parameter. Mean and standard deviation were calculated for each group.

Data for the test substance except immunological parameters were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). Immunological parameters were analyzed by a Kruskal Wallis test. If no significance was found the analysis was stopped, if significance was obtained a two-sided Dunn test was performed.
Data for the positive reference substance (cyclophosphamide) except immunological parameters were analyzed by an F test for the homogeneity of variances. When the data were homogeneous, a two-sided

T test was performed followed by two-sided modified T test on parameters showing a significant effect by the F test. When the data were not homogeneous even after transformation, a two-sided modified T test was performed on transformed data. Immunological parameters were analyzed by Mann-Whitney two-sided test.

C. Methods

1. Daily observations
All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Observed clinical signs were observed at least once daily for the animals exposed to the test substance. Observed clinical signs were recorded at least once daily for animals exposed to the immunosuppressive agent cyclophosphamide. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight
Each animal was weighed at least weekly during the acclimatization period, on the start of treatment (study Day 1), then at weekly intervals throughout the treatment period and before necropsy (terminal body weight).

3. Food consumption
The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period.
The weekly mean achieved dosage intake in mg/kg body weight/day for each week and for Weeks 1 to 4 was calculated except for the group exposed to the immunosuppressive agent cyclophosphamide).

4. Sheep red blood cell (SRBC) sensitization
SRBC characteristics

Identification
Antigen : Sheep Red Blood Cell (SRBC)
Supplier : BioMérieux
Reference number : 72 141

Storage
The SRBC was stored at approximately 5 ± 3 °C.

Activity
SRBC was selected as an appropriate antigen, since it has a large size ensuring proper immunization of animals and since it is recommended by the guideline.

d/Preparation
On the day of injection, Sheep Red Blood Cells were washed in PBS (Phosphate Buffered Saline),

counted using a cell counting instrument (Siemens Advia 120) and diluted in PBS in order to obtain a 5 x 10^8 cells/mL preparation. SRBC preparation was kept on ice until use.

SRBC administration

On Study Day 26 after the start of treatment, all animals in all groups were immunized by intravenous injection in the tail vein (0.5 mL/animal) with Sheep Red Blood Cell (SRBC) preparation. Prior to intravenous injection, animals were anesthetized with Isoflurane (Baxter, Maurepas, France).

5. Clinical pathology

Blood sampling

Blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus 4 days after SRBC immunization (terminal sacrifice). Animals were not diet fasted. Animals were anesthetized by inhalation of Isoflurane (Baxter, Maurepas, France). Blood was placed into tubes with clot activator (for serum preparation). After centrifugation, serum aliquots were frozen (approximately -74 °C) until analysis.

SRBC-specific IgM assay

Enzyme-Linked Immunosorbent Assay (ELISA) was used to determine the level of SRBC-specific immunoglobulin M in response to antigen administration.

Rat Anti-Sheep Red Blood Cell IgM ELISA kits from [redacted] ([redacted]-USA) were used.

Results were obtained using KC4 (version 3.4 Revision 12).

6. Post-mortem examinations

Necrospy

On Study Day 30, all surviving animals from all groups were sacrificed by exsanguination while under deep anesthesia (Isoflurane inhalation).

All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled.

Organ weights

At final sacrifice the following organs were weighed:

- Spleen
- Thymus.

II. RESULTS

A. Mortality

On Study Day 8, one animal administered at 600 ppm with BYI 02960 showed automutilation of both forelimbs and was killed for humane reasons. There were no treatment-related mortalities.

B. Clinical Signs

No treatment-related clinical signs were observed during the study.

C. Body weight

BYI 02960

At 3000 ppm, mean body weight was reduced by 6 to 11% from Study Day 8 to 29 when compared to the control group. The effect was statistically significant on the intervals Day 1 to 8 and Day 8 to 15. There was no body weight gain/day between Study Day 1 and 8. Body weight gain was then similar to control animals at the following intervals. Between Study Day 1 and 29, the cumulative body weight was reduced by 23%, when compared to the control group.
At 600 and 125 ppm, body weight parameters were unaffected by treatment.

D. Food Consumption

BYI 02960

At 3000 ppm, mean food consumption was reduced by approximately 34% on Study Day 8 and by 9 to 13% onwards, when compared to control group although not statistically significant.
At 600 and 125 ppm, body weight parameters were unaffected by treatment.

The mean achieved dose level of BYI 02960 expressed as mg/kg body weight/day received by animals during the study were as follows:

Table 5.10-5: Mean achieved dietary intake of BYI 02960 (Weeks 1 - 4)

Diet concentration (ppm)	Female (mg/kg/day)
125	10
600	50
3000	230

E. SRBC-Specific IgM response

BYI 02960

The high mean anti-SRBC IgM concentration observed in the control group confirmed the sensitization of the animals. A high inter-individual variability was noted in all the groups as usually observed with SRBC sensitization.
No statistically significant difference was noted in anti-SRBC IgM concentrations up to 3000 ppm. No dose-effect relationship was seen, the variations observed were due to only few animals.

Table 5.10-6: Mean SRBC-specific IgM

SRBC-specific IgM (u/mL) mean ± standard deviation (% change when compared to controls)				
BYI 02960 dose level (ppm)	0	125	600	3000
Study Day 30	**40516 ± 23875**	**62920 ± 41649 (+ 55%)**	**34286 ± 30710 (- 15%)**	**33354 ± 26016 (- 18%)**

F. Post-mortem examinations

BYI 02960

1. Terminal body weight and organ weight

At 3000 ppm, a lower mean terminal body weight was observed in treated females when compared to the controls (-6%, not statistically significant).
The few other organ weight changes were considered to be incidental and not treatment-related.

2. Gross pathology

Unscheduled sacrifice

The animal showing automutilation was killed for humane reasons on Study Day 8. No macroscopic finding was noticed.

Terminal sacrifice

All the macroscopic changes were considered as incidental and not treatment-related.

G. Positive control

Homogeneity and concentration of cyclophosphamide formulations ranged from 96 and 99% of the nominal concentration and were therefore within in-house target range of 90 and 110% for a solution.
The toxicological results obtained with the use of the positive control cyclophosphamide at a dose level of 3.5 mg/kg/day were in line with those obtained in previous studies at the test facility.
There was no change in mean terminal body weight in treated animals when compared to the controls.
The principal findings noted at the macroscopic examination were atrophic small spleen and/or thymus (6/10 and 5/10 respectively) which correlated with significantly lower mean absolute and relative spleen and thymus weights (- 34% to - 32% and - 28% to - 27% respectively).

Table 5.10-7: Mean spleen weights

Mean spleen weight ±SD at scheduled sacrifice (% change when compared to controls)		
Sex	**Female**	
Dose level of Cyclophosphamide (mg/kg/day)	0	3.5
Mean absolute spleen weight (g)	0.762 ± 0.122	0.500** ± 0.049 (- 34%)
Mean spleen to body weight ratio (%)	0.3161 ± 0.0380	0.2136** ± 0.0176 (- 32%)

** : $p \leq 0.01$

Table 5.10-8: Mean thymus weights

Mean thymus weight ±SD at scheduled sacrifice (% change when compared to controls)		
Sex	**Females**	
Dose level of Cyclophosphamide (mg/kg/day)	0	3.5
Mean absolute thymus weight (g)	0.544 ± 0.130	0.389** ± 0.063 (- 28%)
Mean thymus to body weight ratio (%)	0.2259 ± 0.0482	0.1656** ± 0.0202 (- 27%)

** : p ≤0.01

At 3.5 mg/kg/day, when compared to controls, mean anti-SRBC IgM concentration was markedly lower when compared to controls (- 93%) corresponding to the range usually observed with cyclophosphamide within our laboratory conditions.
These results confirmed the ability of the test system to detect immuno-suppressive effects and the validity of the study design.

III. CONCLUSION

In conclusion, BYI 02960 was considered not to have any immunotoxic potential in female Wistar rats when given in the diet at dose levels up to 3000 ppm (corresponding to approximately 230 mg/kg/day) for at least 28 days.

Taken into account all systemic effects of BYI 02960, the NOAEL following continuous dietary administration to female Wistar rats for at least 28 days was 600 ppm (equating approximately to 50 mg/kg/day).

Report:	**KIIA 5.10/03, [redacted] D.; 2012**
Title:	BYI 02960 and its major metabolite DFA (difluoroacetic acid) - Toxicological profile comparison
Report No & Document No	Not applicable (position paper) M-428487-01-2
Guidelines:	Not applicable
GLP	Not applicable

Summary

BYI 02960 is a new insecticide being developed by Bayer CropScience; a full toxicological package has been performed for registration purposes. Difluoroacetic acid is a plant and livestock metabolite of BYI 02960 and a major environment metabolite, having been found in soil metabolism studies at ~10% of applied dose. Additionally, DFA is a groundwater metabolite with a PEC gw between 0.75 µg/L and 10 µg/L based on calculations according to EU models. DFA was detected in the rat ADME studies at approximately 6% of the administered dose. However since DFA was not found in the rat ADME studies above 10% of the applied dose, based upon EFSA guidance, select toxicology studies were conducted with DFA to compare its toxicological properties with those of parent compound BYI 02960 and to exclude that DFA is a relevant metabolite according to the EU Guidance document on the assessment of the relevance of metabolite in groundwater (Sanco/221/200 rev. 10). DFA has been tested in an acute oral rat study, *in vitro* genotoxicity studies and in a subchronic rat toxicity study. The purpose of this position paper is to demonstrate that the toxicological profile of DFA is very similar to that of BYI 02960 and DFA should therefore be considered to be covered by the overall toxicological profile of the parent compound.

Acute toxicity studies

BYI 02960 (1) was administered to four groups of three Wistar female rats at 2000 mg/kg and 300 mg/kg (two groups of three animals for each concentration). In the groups treated at 2000 mg/kg, four of six animals died within 2 or 3 hours after administration. The following clinical signs were noted at this concentration: decreased motility, tremors, piloerection, labored breathing and clonical cramps. There was no effect on body weight and no necropsy findings in surviving animals. In the other animals, black liver or liver with black spots and hemorrhagic lung were observed.
No mortality was observed at 300 mg/kg. Clinical signs were limited to breathing sounds. No effects on body weight and no necropsy abnormalities were recorded.

Difluoroacetic acid (2) was administered to three groups of three Sprague-Dawley female rats at 2000 and 300 mg/kg (one group for 2000 mg/kg and two groups for 300 mg/kg). In the group treated at 2000 mg/kg, two of three animals died within 1 hour after administration, showing hypoactivity, dyspnea and/or lateral recumbency within 25 minutes prior to death. Sedation, then hypoactivity, lateral recumbency, dyspnea, piloerection and staggering gait were observed in the surviving animal from day 1 to day 9. Body weight loss of 8% was also observed at the end of the first week, but body weight gain returned to normal thereafter. No abnormality was recorded at necropsy.
No mortality was observed at 300 mg/kg. Clinical signs were limited to loud breathing in one female on day 3. Overall body weight gain was not affected and no abnormalities were recorded at necropsy.

From these two studies it can be concluded that BYI 02960 and difluoroacetic acid have a similar profile for acute toxicity. Both compounds are classified category IV according to the GHS classification system.

Genotoxicity studies

BYI 02960 was tested in four genotoxicity studies: three *in vitro* and one *in vivo* study. All of them were negative. BYI 02960 was tested at doses of up to 5000 µg/plate on Salmonella typhimurium strains TA1535, TA100, TA1537, TA98 and TA102 (3), and at up to 10mM in the HPRT test (4) and the chromosome aberration test (5). In the *in vivo* micronucleus test on NMRI mice (6), three dose levels were tested: 10, 20 and 40 mg/kg (two injections at a 24-hour interval).

Difluoroacetic acid was tested in three *in vitro* studies. As all studies were negative, no further testing was done. Difluoroacetic acid was also tested at up to 5000 µg/plate on Salmonella typhimurium strains TA1535, TA100, TA1537, TA98 and TA102 (7), and at up to 10mM in the HPRT test (8) and the chromosome aberration test (9).

Both compounds are not genotoxic.

Subchronic rat toxicity studies

BYI 02960 was administered to groups of 10 male and 10 female Wistar rats at 100, 500 and 2500 ppm over a period of at least 90 days (10). These dose levels equate to 6, 30 and 156 mg/kg/day in males and 7.6, 38 and 186 mg/kg/day in females. Significant effects were limited to the top dose. A 6% decrease in overall body weight was observed in both males and females. Decreased food consumption was observed mainly during the first week for the males and during the first 7 weeks for the females. A slight increase of 15% in platelet count was observed in the females. Changes were observed in several biochemical parameters at all dose levels but they were only statistically significant at 2500 ppm in both males and females. These changes included a decrease in total bilirubin (- 38% in males and - 45% in females) and glucose (- 21% in males and - 22% in females) and an increase in cholesterol (+ 28% in males and + 46% in females) and in triglycerides (+ 35% in males and + 66% in females).
The target organs were the liver in both males and females and the thyroid in the males only. The slight liver weight increase observed at 2500 ppm in both sexes was mainly due to body weight decrease. However, liver enlargement was observed at necropsy in 4/10 males and 1/10 females. Minimum to slight centrilobular hypertrophy was observed in all males and 3/10 females. Increased thyroid weight of 20 to 26% was obtained in males only at 2500 ppm with minimal follicular cell hypertrophy in 3/10 males. The slight thyroid weight increase of 17 to 20% observed at 500 ppm in males was not considered to be adverse as it was not associated to any histopathological changes. The NOAEL was therefore established at 500 ppm, equating to 30 mg/kg/day for the males and 38 mg/kg/day for the females.

A summary of the findings in this study is presented below in Table 5.10-9.

Table 5.10-9: Significant changes in the 90-day rat study with BYI 02960

Dose levels ppm	Male			Female		
	100	500	2500	100	500	2500
Conc. mg/kg/day	6.0	30.2	156	7.6	38.3	186
Body weight and body weight gain			↓ 6% overall BW ↓ 12% overall BWG Good recovery		↓12% overall BWG	↓ 6% overall BW ↓ 15% overall BWG Good recovery
Food consumption			1st week ↓ 6% Several occ. ↓ 5%			↓ 9% to 29% from week 1 to
Haematology						↑ 15% platelet count, reversible
Clinical chemistry	↓ 15%TBIL NS, ↑ 16%Chol NS ↑ 21%Trigly NS	↓ 15%TBIL NS, ↓ 6%Glc NS, ↑ 10%Chol NS	↓ 38%TBIL **, ↓ 21%Glc **, ↑ 28%Chol NS ↑ 35%Trigly NS reversible	↓ 2%TBIL NS, ↓ 3%Glc NS, ↑ 8%Chol NS	↓10%TBIL NS, ↓ 7%Glc NS, ↑ 20%Chol NS ↑ 9%Trigly NS	↓ 45%TBIL ** ↓ 22%Glc **, ↑ 46%Chol ** ↑ 66%Tgly NS Partially reversible for TBIL ↓ 25%
Organ weights		Thyroid: ↑17 to 20%	Liver: ↑ 8 to 16% mainly due to ↓ BW Thyroid: ↑ 20 to 26%			Liver: ↑ 8 to 15% mainly due to ↓ BW
Macroscopy			Liver: enlargement: 4/10 Thyroid: dark 1/10			Liver: enlargement: 1/10
Histopathology			Liver: minimum to slight centrilobular hypertrophy 10/10 Thyroid: diffuse follicular cell hypertrophy 3/10			Liver: minimum centrilobular hypertrophy 3/10

** : $p \leq 0.01$
NS : Not statistically significant

Difluoroacetic acid was administered to groups of 10 male and 10 female Wistar rats at 200, 1000 and 6000 ppm (11). These dose levels equated to 12.7, 66 and 380 mg/kg/day in males and 15.6, 79 and 472

mg/kg/day in females. A lower body weight was observed in both males and females at 1000 and 6000 ppm as compared to controls. The effect was dose-related in females reaching statistical significance only at the top dose whereas in males it was more marked and statistically significant only at the intermediate dose. A very slight decrease in food consumption was observed at 1000 and 6000 ppm in both sexes. Lower hemoglobin concentration and lower mean corpuscular volume were observed at 1000 and 6000 ppm in females only. As a consequence, lower mean corpuscular hemoglobin and lower hematocrit were also noted, but these changes were observed without a clear dose-effect relationship. Lower mean glucose concentrations were noted at all dose levels in both sexes (from - 29% to - 45% in males and - 27% to - 53% in females). The decrease in glucose was associated with an increase of the urinary ketones. Lower mean total bilirubin concentrations were observed at 6000 and 1000 ppm in both sexes and at 200 ppm in males only (- 27% to - 56% in males and - 37% to - 47% in females). However, in the absence of any signs of systemic toxicity, these changes are not considered to be adverse effects of the test substance as they do not represent any functional impairment in the test organism.
Slightly higher mean urea concentrations were seen at all dose levels in both sexes. There was no dose-effect relationship and as no concomitant change was noted in creatinine concentrations or at the histological examination; therefore, these differences were not considered to be adverse effects. The only target organ was the stomach. At macroscopic examination, black foci were observed in 1/10 males and 2/10 females at 1000 ppm and 3/10 males and 2/10 females at 6000 ppm. This finding was also observed in one female from the control group, which minimizes the significance of this effect. Focal glandular erosion was noted at microscopic examination in 1/9 males and1/10 females at 1000 ppm and 2/10 males and 1/10 females at 6000 ppm. These lesions were graded 'minimal to slight' except the lesion observed in the female at 1000 ppm, which was graded 'moderate'.
The NOAEL in this study was considered to be 200 ppm, equating to 12.7 mg/kg/day in males and 15.6 mg/kg/day in females.

A summary of the findings in this study is presented below in Table 5.10-10.

Table 5.10-10: Significant changes in the 90-day rat study with DFA

Dose levels ppm	Male			Female		
	200	1000	6000	200	1000	6000
Conc. mg/kg/day	12.7	66.2	380	15.6	78.7	472
Body weight and body weight gain		↓ 13% overall BW ** ↓ 20% overall BWG **	↓ 7% overall BW ↓ 11% overall BWG		↓ 6% overall BW ↓ 9% overall BWG	↓ 9% overall BW ** ↓ 20% overall BWG **
Food consumption		↓5% overall	↓5% overall		↓4 overall	↓ 7overall
Haematology					↓ 9% [Hg] ** ↓ 6% MCV ** ↓ 8% MCHg ** ↓ 7% Ht **	↓ 8% [Hg] ** ↓ 7% MCV ** ↓ 7% MCHg ** ↓ 6% Ht **
Clinical chemistry	↓ 27%TBIL * ↓ 29%Glc ** ↑ 15% Urea *	↓ 52%TBIL ** ↓ 45%Glc ** ↑ 26% Urea **	↓ 56%TBIL ** ↓ 45%Glc ** ↑ 18% Urea *	↓ 12%TBIL NS ↓ 27%Glc ** ↑ 14% Urea *	↓ 37%TBIL ** ↓ 49%Glc ** ↑ 23% Urea **	↓ 47%TBIL ** ↓ 53%Glc ** ↑ 12% Urea NS
Urinalysis : urine volume	↑ 112% NS	↑ 240% ***	↑ 266% ***	↑ 28% NS	↑ 105% NS	↑ 155% **
Urinalysis: Ketones	Grade 0:0 Grade 1:2 Grade 2:8 Grade 3:0 Grade 4:0	Grade 0:0 Grade 1:0 Grade 2:0 Grade 3:0 Grade 4:10	Grade 0:0 Grade 1:0 Grade 2:0 Grade 3:0 Grade 4:10	Grade 0:6 Grade 1:2 Grade 2:0 Grade 3:0 Grade 4:0	Grade 0:0 Grade 1:0 Grade 2:5 Grade 3:4 Grade 4:0	Grade 0:0 Grade 1:0 Grade 2:0 Grade 3:7 Grade 4:2
Macroscopic evaluation		Stomach: black foci 1/10	Stomach: black foci 3/10		Stomach: black foci 2/10	Stomach: black foci 2/10
Histopathology		Stomach: focal glandular erosion 1/9 (minimal)	Stomach: focal glandular erosion 2/10 (minimal & slight)		Stomach: focal glandular erosion 1/10 (moderate)	Stomach: focal glandular erosion 1/10 (slight)

* = p≤ 0.05 ** = p≤ 0.01 *** = p ≤0.001

Conclusion

Although slightly different findings were observed in each of the 90-day rat studies, the overall toxicological profile is very similar. Therefore, we consider that difluoroacetic acid is covered by the toxicological endpoints obtained with the parent compound, BYI 02960. The compounds are of similar acute toxicity, although no clinical signs or neurotoxic effects were observed after difluoroacetic acid administration. The metabolic changes observed with difluoroacetic acid were also observed with

BYI 02960 in a 28-day rat study (12) at 5000 ppm, where a marked decrease in total bilirubin (- 73%) and glucose (- 46%) and a significant increase in urea (+ 37%) and total cholesterol (+ 41%) compared to controls were observed at 5000 ppm. The most significant effect detected is the decrease in glucose, which however did not cause any change in the behavior of the animals nor did it cause any functional impairment. Furthermore, a decrease in glucose was also observed in the parent 90-day study after administration of BYI 02960 at 2500 ppm and this decrease in glucose was reversible as no significant change was observed at the end of a 28-day recovery period. Also, in the 2-year rat study, a decrease in glucose was observed in both males and females treated at 2000 ppm after 3 months of administration (- 23% in males and - 18% in females) and in males only after 6 months (- 16%) but no significant variations were observed thereafter (13). Therefore, the effect on glucose should be considered adaptative as demonstrated in the combined rat chronic toxicity and carcinogenicity study. The effects observed in the stomach after DFA administration are very minor lesions which could be due to stress and which are only seen at quite high dose levels and with very low incidence.

In conclusion, no separate risk assessment for difluoroacetic acid is considered necessary. Risk assessment can be performed on the basis of total residue (as defined in the residue definition) and based upon parent ADI and ARfD.

Even if the toxicological profile of difluoroacetic acid would not be considered to be covered by the toxicological profile of BYI 02960, the ADI calculated for difluoroacetic acid based on two alternative scenarios is above or very close to the parent ADI, confirming that a separate risk assessment for difluoroacetic acid is not warranted. The two scenarios are presented below.

In the first scenario, the clinical chemistry changes observed at the low dose in the 90-day rat toxicity study are considered to be non-adverse in the absence of other evidence of systemic toxicity. Therefore the ADI for difluoroacetic acid could be calculated from the NOAEL of the 90-day rat study using the different default extrapolation factors proposed by EFSA (Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data, EFSA Journal 2012;10(3):2579):

NOAEL = 200 ppm (12.7/15.6 mg/kg/day)
Extrapolation from subchronic to chronic duration: uncertainty factor = 2
Default inter-/intra-species extrapolation: uncertainty factor =100

ADI = 12.7 /2 x 100 = 0.065 mg of DFA/kg bw/day
Corresponding to an ADI of 0.195 mg BYI 02960 equivalent/kg bw/day.

The ADI derived for difluoroacetic acid is less critical than the ADI derived from parent (0.078 mg/kg bw/day derived from the rat 2-generation reproduction study based on body weight effects in parental females). Therefore the single risk assessment performed on the basis of total residue data and parent ADI is even more conservative than performing separate assessments for parent compound and DFA.

In the second scenario, the low dose of 200 ppm is not considered as a NOAEL in the 90-day rat study. As recommended by EFSA (EFSA Scientific Committee, 2009), it is then preferable to use the bench

mark dose (BMD) approach instead of applying an additional uncertainty factor to the LOAEL. Thus, the lower confidence limit of bench mark dose (BMDL) has been calculated based on the glucose data from the 90-day rat study (see attachment 1). The combined data set used to calculate the BMD included combined glucose values for males and females at all doses and dropping the highest dose for males and females. The best models were the exponential models 4 and 5 and the Hill model. Based on the AIC criteria, the Hill model is the most appropriate with a BMD of 2.99 mg/kg/day and a BMDL of 2.07 mg/kg/day. Using the same EFSA guidance document on default values, the ADI for difluoroacetic acid could be calculated as follows:

BMDL = 2.07 mg/kg bw/day
Extrapolation from subchronic to chronic duration: no uncertainty factor needed
Default inter-/intra-species extrapolation: uncertainty factor = 100

ADI = 2.07 / 100 = 0.0207 mg DFA/kg bw/day
Corresponding to an ADI of 0.062 mg of active substance equivalents/kg bw/day.

The ADI derived for difluoroacetic acid is close to the ADI derived for parent (0.078mg/kg bw/day). Therefore risk assessment could still be performed from total residue data and parent ADI.

In the case, the BMDL (2.94) calculated with the exponential models 4 and 5 is used, the ADI is even above the ADI derived for parent:

BMDL = 2.94 mg/kg/day
Extrapolation from subchronic to chronic duration: no uncertainty factor needed
Default inter-/intra-species extrapolation: uncertainty factor =100

ADI = 2.94 / 100 = 0.0294 mg DFA/kg bw/day
Corresponding to an ADI of 0.088 mg of active substance equivalents/kg bw/day.

The ADI derived for difluoroacetic acid is above the ADI derived for parent (0.078mg/kg bw/day). Therefore risk assessment based on total residue data and parent ADI would be more conservative.

In conclusion, we consider that the toxicological profile of DFA is similar to the toxicological profile of BYI 02960. The endpoints derived from the parent database should be used for risk assessment.

Report:	**KIIA 5.10/04, [redacted]; 2012**
Title:	Toxicological coverage of BYI 02960 plant and livestock metabolites
Report No & Document No	Not applicable (position paper) M-428863-01-2
Guidelines:	Not applicable
GLP	Not applicable

Remark:

The position paper was written for the submission of fluopyradifurone in Europe. As a consequence the uses are not identical with the uses submitted in this dossier. However the principle of the concept described can be applied.
The following section estimates the metabolite concentrations in all registered crops according to the critical use patterns presented in in this dossier.

Background

The parent compound BYI 02960 shows a quite extensive metabolic behaviour in crops, and also in livestock, whereas metabolization was less pronounced in the rat. Thus several plant and/or livestock metabolites were not detected as systemic metabolites in the rat ADME studies. To assess the possible health risk due to the dietary exposure to these metabolites, a new conservative risk assessment approach has been applied on the basis of the scientific report "Impact of metabolic and degradation processes on the toxicological properties of residues of pesticides in food commodities" prepared by the Institute for Plant Protection Product Evaluation and Authorisation of the Austrian Agency (AGES, 2010), and the EFSA scientific opinion on "Exploring options for providing advice about possible human health risks based on the concept of Threshold of Toxicological Concern (TTC)". These scientific reports refer to a decision tree which allows the evaluation of the human safety on basis of the exposure to a metabolite.
According to this approach no additional toxicity data are needed for a metabolite, if

(1) the dietary metabolite was detected as systemic metabolite in the rat ADME study and accounted for >10% of the administered dose,
(2) the metabolite shows structural similarities to the parent/precursor and if the metabolic pathway from parent/precursor to the questioned metabolite is simple and it is unlikely that the metabolization step causes toxification (e.g. demethylation, hydroxylation of a ring system),
(3) the consumer is not exposed to this metabolite, i.e. that it accounts for <0.01 mg/kg in food items and <0.05 mg/kg in feed items, or
(4) the metabolite is considered not to be geno- or neurotoxic (based on QSAR analysis using e.g. DEREK) and the consumer exposure is assumed to be below the threshold of toxicological concern (TTC) for Cramer Class III compounds (<1.5 µg/kg bw/day, or 90 µg/person/day for chronic assessment and <5.0 µg/kg bw/day, or 300 µg/person/day for acute assessment).

Evaluation of the toxicological coverage of BYI 02960 metabolites

(1) Dietary metabolites covered by the rat ADME studies

BYI 02960 was moderately metabolised in the rat. The parent compound represented the predominant part of the radioactivity in urine (the preferred route of excretion) of male and female rats. The main metabolite detected in urine and faeces was BYI 02960-OH, which accounted for 11% to 29% of the dose administered in excreta. Thus BYI 02960-OH was present in sufficient amount to contribute to the toxicological effects when testing the parent compound.
The two metabolites, 6-CNA (<1% to 6%) and BYI 02960-hippuric acid (1% to 11%) were also prominent in male but not in females rats. In sum the metabolites represented 10% to 16% of the administered dose in male rats. Since BYI 02960-hippuric acid can be formed from 6-CNA only, the proportion of BYI 02960-hippuric acid can be added to the 6-CNA proportion. Thus, when considering the two metabolites together, 6-CNA accounted for ≥10% of the administered dose in male rats. Since there is no sex difference in the toxicological profile of BYI 02960, it can be concluded that the proportion of 6-CNA in rat excreta is adequately addressed. Thus 6-CNA was present in a sufficient concentration to contribute to the toxicological effects when testing the parent compound.
All other metabolites detected in the rat ADME represented less than 5% of the administered dose.

(2) Metabolites showing structural similarity to parent/precursors and arising from metabolisation steps which will cause no toxification

Conjugation of metabolites is in general a detoxification step. Thus it can be considered that the toxicity of a conjugate is covered by its aglycon. Therefore, the toxicity of the conjugates of BYI 02960-OH (isomers of BYI 02960-OH-gluA, BYI 02960-OH-SA, BYI 02960-OH-glyc, isomers of BYI 02960-OH-glyc-SA) and of 6-CNA (6-CNA-glycerol-gluA) detected in plants and livestock can be considered as toxicologically covered.

(3) Metabolites showing no consumer exposure

METABOLITES IN ANIMAL MATRICES

Based on the residues levels detected in the livestock metabolism studies, it was assumed that most of the metabolites detected show no or only low consumer exposure under real conditions. The livestock metabolism studies were conducted with a dose level of 1 mg/kg bw/day, which represents an overdose compared to the real exposure. The actual residue intake of livestock was calculated with the OECD dietary burden calculator based on the parent BYI 02960 residue levels determined in the North American supervised residue trials as shown in the following tables. All crops intended for this submission are considered.

Livestock dietary intake:

Table 5.10-11: Input data used for dietary burden calculation (BYI 02960 residues, only)

Crop	Feedstuff	DM	Type	BYI 02960 residue [mg/kg]	Remark
alfalfa	forage	35	HR	8.7	
alfalfa	hay	89	HR	11	
barley	hay	88	HR	26	
barley	straw	98	HR	5.8	
clover	forage	30	HR	6.4	
clover	hay	89	HR	12	
corn, field	forage/silage	40	HR	4.1	
corn, field	stover	83	HR	6.1	
pea	vines	25	HR	6.2	
pea	hay	88	HR	18	
soybean	hay	85	HR	20	
wheat	forage	25	HR	15	
wheat	straw	88	HR	20	
carrot	culls	12	HR	1.0	
potato	culls	20	HR	0.06	
barley	grain	88	STMR	0.48	
corn, field	grain	88	STMR	0.01	
cotton	undelinted seed	88	STMR	0.12	
pea	seed	90	STMR	0.64	
sorghum	grain	86	STMR	0.56	
soybean	seed	89	STMR	1.1	
wheat	grain	89	STMR	0.17	
almond	hulls	90	STMR	2.6	
apple	pomace, wet	40	STMR-p	0.22	STMR * PF = 0.15 * 1.45
citrus	dried pulp	91	STMR-p	1.6	STMR * PF = 0.34 * 4.75
corn, field	aspirated grain	85	STMR-p	0.23	STMR * PF = 0.01 * 23.4
cotton	meal	89	STMR-p	0.02	STMR * PF = 0.12 * 0.14
cotton	hulls	90	STMR-p	0.06	STMR * PF = 0.12 * 0.52
cotton	gin by-product	90	STMR	10	
soybean	aspirated grain fraction	85	STMR-p	15.4	STMR * PF = 1.1 * 14
soybean	meal	92	STMR-p	1.1	STMR * PF = 1.1 * 1.0
soybean	hulls	90	STMR-p	1.1	STMR * PF = 1.1 * 0.95
wheat	aspirated grain fraction	85	STMR-p	12.2	STMR * PF = 0.68 * 18
wheat gluten	meal	40	STMR-p	0.24	STMR * PF = 0.68 * 0.35

STMR = supervised trial median residue
PF = processing factor (based on parent compound residues)

Ruminants:

Table 5.10-12: Estimated dietary intake for dairy cattle according to the OECD dietary intake calculator

Cattle - Dairy	**US/CAN**	
(= worst-case)	**Body weight (kg)**	**600**
	Daily feed intake (kg DM)	**24**

Feedstuff type	Crop	Feedstuff	Maximum Percent of Diet	Dietary burden (mg/kg bw/day)
R	barley	hay	20	0.236
R	wheat	forage	20	0.480
R	alfalfa	forage	5	0.050
CC	sorghum	grain	35	0.009
CC	carrot	culls	10	0.033
PC	soybean	seed	10	0.005
Total			**100**	**0.813**

Residue level in total feed dry matter (mg/kg)	20.337
Residue intake (mg/animal/day)	488.083
Residue intake (mg/kg bw/day)	0.813

Comparing the worst-case dietary intake for dairy cattle (0.081 mg/kg bw/day), estimated on basis of supervised residue trials conducted according to the critical GAPs, with the dose administered in the lactating goat metabolism studies (1 mg/kg bw/day), it can be shown that the metabolism studies were overdosed by a factor of approx. 1.2. Considering this slight overdose, only metabolite BYI 02960-methylthio-glyoxylic acid will not exceed a residue level of 0.01 mg/kg under realistic conditions. However, the highest residues in the goat matrices were estimated for parent compound, metabolite BYI 02960-OH and its glucuronic acid conjugates. BYI 02960-OH was detected in the rat ADME study, as well as two isomers of its glucuronic acid conjugate and thus a toxicological coverage is given. All other metabolites – except BYI 02960-hippuric acid, which is also a major rat metabolite - were detected in the metabolising organs kidney and liver, which do not represent a major part of the human diet and thus exposure is low. Moreover, BYI 02960-cysteinyl-nicotinic acid is also covered by the toxicity profile of the parent. The chloro atom of the pyridinyl moiety is substituted by the amino acid cysteine which is further conjugated with nicotinic acid. Both compounds occur naturally in the human body. Also the natural compound lactose is not of toxicological concern (cf. also point (6) in this section). Thus only the metabolites BYI 02960-AMCP-difluoroethanamine (which is also a plant metabolite) and BYI 02960-desdifluoroethyl have to be evaluated against the TTC threshold level of 1.5 µg/kg bw/day for chronic exposure.

The following tables summarise the metabolite concentrations in milk and the edible organs/tissues of lactating goats after five consecutive administrations of 1 mg parent compound per kg bw/day. The

actual metabolite concentration arising from the use of flupyradifurone on ruminant feed items can be estimated by dividing the concentrations given by a factor of 1.2.

Table 5.10-14: Distribution of parent compound and metabolites in edible matrices of a lactating goat after five administrations of 1 mg [pyridinylmethyl-^{14}C]BYI 02960 per kg bw
(cf. 2.1.2.1/01; Table 2.1.2.1-3)

Sample	**Milk (24 to 102 h)**		**Muscle**		**Fat**		**Kidney**		**Liver**	
TRR	**0.186**		**0.356**		**0.106**		**1.869**		**1.215**	
Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
cysteinyl-nicotinic acid	---	---	---	---	---	---	6.1	0.114	4.8	0.058
hippuric acid	9.1	0.017	---	---	---	---	9.5	0.178	0.8	0.010
methylthio-glyoxylic acid	1.5	0.003	1.3	0.005	---	---	---	---	---	---
OH-gluA (I 1)	---	---	---	---	---	---	6.0	0.112	---	---
OH-gluA (I 2)	---	---	---	---	---	---	9.3	0.175	1.4	0.016
OH-gluA (I 3)	---	---	---	---	---	---	8.4	0.158	---	---
OH-gluA (I 4)	---	---	---	---	---	---	7.5	0.141	---	---
AMCP-difluoroethanamine	---	---	---	---	---	---	1.1	0.020	1.2	0.015
OH							16.0	0.299		
parent compound	88.8	0.165	98.0	0.349	99.2	0.105	34.8	0.650	84.6	1.028
Total identified	**99.3**	**0.184**	**99.4**	**0.353**	**99.2**	**0.105**	**98.8**	**1.847**	**92.8**	**1.128**
Not analysed	0.1	<0.001	0.1	<0.001	0.6	0.001	0.1	0.001	0.1	0.001
Total extracted	99.5	0.185	99.5	0.354	99.8	0.106	98.9	1.848	92.9	1.129
Solids	0.5	0.001	0.5	0.002	0.2	0.001	1.1	0.021	7.1	0.086
Accountability	**100.0**	**0.186**	**100.0**	**0.356**	**100.0**	**0.106**	**100.0**	**1.869**	**100.0**	**1.215**

I = isomer; I 1 to I 4 = 4 isomers of BYI 02960-OH-gluA

Remark: Deviations in "Total identified" arise from rounding

Table 5.10-15: Distribution of parent compound and metabolites in edible matrices of a lactating goat after five administrations of 1 mg [furanone-4-^{14}C]BYI 02960 per kg bw
(cf. 2.1.2.1/02; Table 2.1.2.1-6)

Sample	**Milk (24 to 102 h)**		**Muscle**		**Fat**		**Kidney**		**Liver**	
TRR	**1.046**		**0.539**		**0.265**		**1.472**		**1.746**	
Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
lactose	66.8	0.698	---	---	---	---	---	---	---	---
OH-gluA (I 1)	---	---	---	---	---	---	2.2	0.032	---	---
OH-gluA (I 2)	---	---	---	---	---	---	2.2	0.032	---	---
OH-gluA (I 3)	---	---	---	---	---	---	4.7	0.069	---	---
OH-gluA (I 4)	---	---	---	---	---	---	3.5	0.052	---	---
des-difluoroethyl	---	---	---	---	---	---	1.3	0.019	---	---
OH	---	---	1.8	0.010	2.9	0.008	14.6	0.215	---	---
parent compound	23.9	0.250	88.1	0.475	80.5	0.213	50.5	0.744	59.8	1.045
Total identified	**90.7**	**0.948**	**89.9**	**0.484**	**83.4**	**0.221**	**79.0**	**1.163**	**59.8**	**1.045**
Total charact.	**---**	**---**	**5.1**	**0.028**	**5.0**	**0.013**	**10.0**	**0.148**	**13.5**	**0.235**
Not analysed	0.7	0.008	0.2	0.001	5.6	0.015	11.0	0.161	26.8	0.466
Total extracted	91.4	0.956	95.2	0.513	94.1	0.249	100.0	1.472	100.0	1.746
Solids	8.6	0.090	4.8	0.026	5.9	0.016	---	---	---	---
Accountability	**100.0**	**1.046**	**100.0**	**0.539**	**100.0**	**0.265**	**100.0**	**1.472**	**100.0**	**1.746**

I = isomer; I 1 to I 4 = 4 isomers of BYI 02960-OH-gluA
Remark: Deviations in "Total identified" arise from rounding

Poultry:

Table 5.10-16: Estimated dietary intake for poultry (broiler) according to the OECD dietary intake calculator

Poultry, Broiler (worst-case)	**EU**	
	Body weight (kg)	**2.0**
	Daily feed intake (kg)	**0.16**

Feedstuff type	**Crop**	**Feedstuff**	**Maximum Percent of Diet**	**Dietary burden (mg/kg bw/day)**
CC	sorghum	grain	75	0.039
CC	barley	grain	10	0.004
PC	soybean	seed	15	0.015
Total			**100**	**0.058**

Residue level in total feed dry matter (mg/kg)	0.728
Residue intake (mg/animal/day)	0.117
Residue intake (mg/kg bw/day)	0.058

Comparing the worst case dietary intake for poultry (0.058 mg/kg bw/day), estimated on basis of supervised residue trials conducted according to the critical GAPs, with the dose administered in the laying hen metabolism studies (1 mg a.s./kg bw/day), it can be calculated that the metabolism studies were overdosed by a factor of approx. 17. Thus, none of the metabolites detected in the samples of the metabolism studies would exceed a residue level of 0.010 mg/kg under real conditions, except for the fatty acids which were detected in high concentrations in eggs, fat and liver. However since fatty acids as natural compounds do not exhibit any toxicological concern, these metabolites have not further to be considered (cf. point (6) in this section concerning "metabolites of no toxicological concern"). As for the lactating goat studies, the highest amounts of metabolites were detected in the metabolizing organ liver, which does not represent a major part of the human diet. In eggs, muscle and fat all xenobiotic metabolites were estimated to account for less than 0.001 mg/kg.

The following tables summarise the metabolite concentrations in eggs and the edible organs/tissues of laying hens after fourteen consecutive administrations of 1 mg a.s./kg bw/day. The actual metabolite concentration arising from the use of flupyradifurone on poultry feed items can be estimated by dividing the concentrations given by a factor of 17.

Table 5.10-18 Distribution of parent compound and metabolites in edible matrices of a laying hen after fourteen administrations of 1 mg [pyridinylmethyl-^{14}C]BYI 02960 per kg bw
(cf. 2.1.2.2/01, Table 2.1.2.2-4)

Sample	Eggs (day 3 to day 13)		Muscle		Fat		Liver	
TRR	**0.084**		**0.070**		**0.021**		**0.435**	
Report name BYI 0260-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
lactato-mercaptyl-nicotinic acid	4.0	0.003	3.6	0.002	---	---	15.5	0.068
acetyl-cysteinyl-nicotinic acid	---	---	---	---	---	---	0.3	0.001
6-CNA	7.2	0.006	8.8	0.006	1.8	<0.001	6.4	0.028
des-difluoroethyl-OH-SA	---	---	2.1	0.001	5.6	0.001	3.1	0.014
acetyl-AMCP	23.1	0.019	40.2	0.028	28.5	0.006	6.3	0.027
des-difluoroethyl	8.9	0.007	9.9	0.007	5.0	0.001	1.8	0.008
AMCP-difluoroethanamine-SA	---	---	---	---	---	---	0.3	0.001
OH-SA	5.1	0.004	1.8	0.001	16.2	0.003	22.5	0.098
OH	18.0	0.015	8.1	0.006	5.5	0.001	1.5	0.007
parent compound	19.8	0.017	9.8	0.007	15.3	0.003	0.9	0.004
Total identified	**86.2**	**0.072**	**84.2**	**0.059**	**77.9**	**0.016**	**58.6**	**0.255**
Total characterised	**9.9**	**0.008**	**8.4**	**0.006**	**1.8**	**<0.001**	**35.8**	**0.156**
Not analysed	---	---	---	---	---	---	---	---
Total extracted	96.1	0.081	92.6	0.064	79.7	0.017	94.5	0.411
Solids	3.9	0.003	7.4	0.005	20.3	0.004	5.5	0.024
Accountability	**100.0**	**0.084**	**100.0**	**0.070**	**100.0**	**0.021**	**100.0**	**0.435**

Table 5.10-19: Distribution of parent compound and metabolites in edible matrices of a laying hen after fourteen administrations of 1 mg [furanone-4-^{14}C]BYI 02960 per kg bw
(cf. 2.1.2.2/01, Table 2.1.2.2-8)

Sample	**Eggs (day 2 to 7)**		**Eggs (day 8 to 13)**		**Muscle**		**Fat**		**Liver**	
TRR	**0.540**		**1.048**		**0.183**		**0.427**		**2.178**	
Report name BYI 0260-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
fatty acids	52.0	0.281	58.3	0.611	8.1	0.015	98.5	0.421	51.5	1.121
des-difluoroethyl-OH-SA	0.1	0.001	---	---	0.5	0.005	---	---	0.2	0.004
desdifluoroethyl	1.2	0.006	0.6	0.007	2.6	0.005	---	---	0.8	0.017
OH-SA	0.6	0.003	0.5	0.005	---	---	---	---	5.1	0.112
OH	2.3	0.013	1.6	0.016	2.4	0.004	---	---	0.8	0.018
parent compound	2.3	0.013	1.6	0.016	2.9	0.005	---	---	0.5	0.010
Total identified	**58.5**	**0.316**	**62.5**	**0.656**	**16.5**	**0.030**	**95.9**	**0.410**	**58.9**	**1.282**
Total charact.	**22.9**	**0.124**	**21.2**	**0.223**	**69.7**	**0.128**	**2.6**	**0.011**	**34.9**	**0.761**
Not analysed	17.5	0.094	14.7	0.155	10.3	0.019	---	---	4.5	0.098
Total extracted	81.5	0.440	83.8	0.878	86.2	0.158	98.5	0.421	93.8	2.044
Solids	1.1	0.006	1.5	0.016	3.5	0.006	1.5	0.006	1.7	0.036
Accountability	**100.0**	**0.540**	**100.0**	**1.048**	**100.0**	**0.183**	**100.0**	**0.427**	**100.0**	**2.178**

Considering these estimations, no consumer exposure is given for the dietary metabolites of poultry origin.

Estimation of a realistic dietary exposure to **BYI 02960-desdifluoroethyl**

BYI 02960-desdifluoroethyl is a metabolite which was only detected in livestock matrices. Comparing the doses applied in the metabolism studies to a realistic dietary burden of the animals, it becomes obvious that the metabolite can be only expected in kidney of ruminants. Due to the low significance of kidney in the human diet, the international estimated daily intake (IEDI) will be below 1% of the threshold level of 1.5 µg/kg bw/day, and is thus negligible. Based on this estimation, it can be concluded that the exposure to the metabolite is so low that the probability of adverse health effects is negligible and no further data are considered necessary.

METABOLITES IN PLANT MATRICES

BYI 02960-difluoroethyl-OH-glyc is a metabolite which was identified in only one edible matrix in all plant metabolism studies: Apple fruits of the single application experiment showed minor concentrations of this metabolite, whereas it was not detected in the double application experiment. When estimating the metabolite concentration on basis of the highest parent residue in the field residue trials, it can be shown that the metabolite will not exceed 0.01 mg/kg in fruits and thus no consumer exposure is given.

Calculation:

Estimated residue (metabolite) = HR (marker) in field trial / ratio (marker/metabolite) in metabolism study

Residues detected in apple metabolism study (pyridinylmethyl-label, single application experiment):

	residues in metab. study [mg a.s. equiv./kg]		**ratio**
	-difluoroethyl-OH-glyc	marker compound	marker / metabolite
apple fruit	0.001	0.034	34.0

Estimated residue = HR (marker) in field trial / ratio (marker/metabolite) in metabolism study

crop matrix	**ratio used**	**residues in field studies [mg a.s.equiv./kg]**	
		marker compound	estimated residue
apple fruit	34.0	0.30 (a.s.)	0.009

⇨ estimated dietary exposure of metabolite BYI 02960-difluoroethyl-OH-glyc is below the trigger value of 0.01 mg/kg

(4) Metabolites with a consumer exposure below the threshold of toxicological concern

The threshold of toxicological concern (TTC) is based on the concept that there are levels of exposure to chemicals that will result in no appreciable risk to health. The classification of chemicals according to their chemical structure is an important component of the current TTC approach. The classification scheme most widely used is that described by [redacted] et al. (1978). Plant metabolites and degradates of pesticide active substances are assigned to Cramer Class III. The corresponding dietary exposure threshold is **1.5 µg/kg body weight per day** for substances with no structural alerts for genotoxicity and neurotoxicity. Neonicotinoids and pyrethroids are included in Cramer Class III according to the Scientific Report "Applicability of thresholds of toxicological concern in the dietary risk assessment of metabolites, degradation and reaction products of pesticides", published by CRD in 2009. There is no reason to include them in the neurotoxic TTC grouping, with a threshold value of 0.3 µg/kg body weight per day.

In accordance with this concept, a two-step approach was applied for all BYI 02960 metabolites for which a consumer exposure was expected and no toxicological coverage was proven yet (e.g. by the rat ADME studies or additional toxicological tests).

(1) Exclusion of genotoxicity:
Each metabolite was tested for genotoxic alerts by DEREK. DEREK is a commonly used *in silico* expert system for the qualitative prediction of toxicity.
In addition metabolites being representative for a metabolic path have been tested for genotoxicity, i.e. negative genotoxicity tests are available for
- parent compound BYI 02960
- DFA
- DFEAF
- CHMP
- 6-CNA
- BYI 02960-acetic acid
- BYI 02960-amino-furanone

(2) Estimation of the possible consumer exposure
Based on the International Estimated Daily Intake (IEDI) Model as described on the WHO web page (http://www.who.int/foodsafety/areas_work/chemical-risks/gems-food/en/), the possible consumer exposure of a metabolite or a group of metabolites was estimated. Conjugates and their aglycons were considered as one metabolite group and their concentrations (in mg a.s. equiv./kg) were summed up for a combined evaluation. Metabolites resulting from the same metabolic path were also considered as a metabolite group.

The following input parameters were used:
- The threshold level of 0.0015 mg/kg bw/day was set as ADI.
- The residue concentration of a metabolite or a group of metabolites in a food item was estimated based on the concentration of a marker compound (generally parent compound and in some cases DFA) in the field samples collected in residue trials conducted in North America (for primary crops) or in Europe (for rotational crops).

(a) Food items of plant origin
The residue value was estimated as follows:
1 Parent-to-metabolite ratio was calculated in the corresponding metabolism study

2 The metabolite concentration in the food item was estimated on basis of the parent concentration determined in the field residue or field rotational crop trials
HR(metabolite) = HR(parent) / ratio (parent/metabolite)

If the field studies showed parent concentrations below the LOQ and DFA concentrations above the LOQ, DFA was used as the marker compound to estimate the metabolite concentration:
HR(metabolite) = HR(DFA) / ratio (DFA/metabolite)

If a metabolite was common to both radiolabels tested, the more critical residue value was used in the subsequent calculation steps.

If the use of HR values resulted in an exceedance of the threshold value, a refinement was possible by using the STMR values (STMR values are more appropriate to describe chronic exposure, however the first tier calculation was always done with the HR values. If the chronic risk assessment is passed with the HR values, an acute risk can be excluded as well.

(b) Food items of animal origin
The residue value was estimated as follows:

1 Parent-to-metabolite ratio in a livestock feed item was calculated in the corresponding metabolism study
2 The metabolite concentration in a field sample was estimated on basis of the parent concentration determined in the field residue trials (NAFTA trials) or the field rotational crop trials (EU trials):
HR(metabolite) = HR(parent) / ratio (parent/metabolite) or
STMR(metabolite) = STMR(parent) / ratio (parent/metabolite)
If the field studies showed parent concentrations below the LOQ and DFA concentrations above the LOQ, DFA was used as the marker compound to estimate the metabolite concentration:
HR(metabolite) = HR(DFA) / ratio (DFA/metabolite) or
STMR(metabolite) = STMR(DFA) / ratio (DFA/metabolite)

If a metabolite was common to both radiolabels tested, the more critical residue value was used in the subsequent calculation steps.

3 The dietary intake of the metabolite by farm animals was calculated according to the OECD table of feedstuff.
4 The residues in food items of animal origin (milk, meat, eggs, fat, kidney and liver) were estimated by using the worst-case transfer factors derived for the total residue in the feeding studies.

Remark: Only the feed items were considered which will result in the maximum dietary burden for ruminants and poultry.

The dietary exposure of the following metabolites or group of metabolites was estimated:

(1) BYI 02960-bromo/-chloro, BYI 02960-bromo-amino-furanone and BYI 02960-amino-furanone
(2) BYI 02960-mercapto-lactic acid
(3) BYI 02960-acetic acid and -acetic acid-glyc
(4) BYI 02960-glyoxylic acid, BYI 02960-AMCP-difluoroethanamine and BYI 02960-N-formyl-/ BYI 02960-N-acetyl-AMCP-difluoroethanamine

The following tables summarize the basic data used for the estimation of the dietary exposure of these metabolites.

- Table 5.10-20 summarizes the metabolism studies which were used as representative studies to estimate the field residue concentration in a crop for which a BYI 02960 use is proposed in this dossier
- Table 5.10-21 summarizes the residue levels of the marker compounds in the field residue studies of the different crops. For feed items, the crop matrices were considered which will contribute to the maximum dietary burden of ruminants and poultry.
- Table 5.10-22 summarizes the transfer factors used to calculate the dietary burden in food items of animal origin. The transfer factors have been determined in the poultry and cattle feeding studies (7.1/01 and 7.2/01). Transfer factors were estimated for the total residue of BYI 02960 (sum of BYI 02960 and DFA = total residue for risk assessment). The transfer factors calculated based on the residues in the animal matrices of cattle were in the same range for all doses administered, since residues increased proportionally with the dose rate. In the poultry feeding study, higher transfer of residues was determined at lower doses; thus residues in animal matrices were not linearly dependent from the dose administered. Thus for poultry matrices, transfer factors of the lowest dose were used to cover the most critical scenario and for cattle matrices, transfer factors calculated for the highest dose, were used (see Table 5.10-22).

Table 5.10-20: Metabolism studies used as a basis to calculate metabolite concentrations in crops in which a use of BYI 02960 is proposed

		Representative for the following	
Metabolism study	**Application technique**	**Crop group**	**Crop**
apple (2 x 75 g a.s./ha/mCH)	foliar spray	fruiting crops	- citrus - pome fruit - blueberries - grape - strawberries - prickly pear cactus - cucumber / zucchini - melon - summer squash - tomato / eggplant - bell pepper - non-bell (chili) pepper - sweet corn
		leafy crop	- cabbages - mustard green - lettuce - spinach
		stalk vegetable	- celery
		legume vegetables	- fresh beans and peas
cotton (2 x 200 g a.s./ha)	foliar spray	oilseeds	- almond - pecan nut - peanut - soybean - cotton - coffee
cotton intermediate (1 x 200 g a.s./ha)		leafy crops[2]	- hops
rice (2 x 200 g a.s./ha)	foliar spray	cereals	- barley - corn - sorghum - wheat
potato (1 x 626 g a.s./ha)	in-furrow application	root crops	- potato (tuber vegetable) - carrots
		bulb vegetables[3]	- onions
		pulses[3]	- dry beans and peas (pulses)
confined rotational crops (1 x 400 g a.s./ha)	application on bare soil plant back interval: 29 days	cereal feed commodities	- wheat forage - barley hay
		legume animal feed[4]	- alfalfa forage

1 Apple was used as surrogate since no metabolism study was conducted in leafy crops, stalk or legume vegetables (2 spray applications)

2 Cotton intermediate was used as surrogate since no metabolism study was conducted in leafy crops (1 spray application)

3 Potatoes were used as surrogate since no metabolism study was conducted in bulb vegetables or pulses

4 Cereals forage from the rotational crop study was used as surrogate since no metabolism study was conducted in a leafy crop

Table 5.10-21: Residue levels of the marker compounds detected in the field residue trials

Crop matrix	Marker compound	Residue level [mg/kg]	
Residues after two foliar spray applications		**HR**	**STMR**
Orange (IT)	BYI 02960	2.1	0.31
Grapefruit (IT)	BYI 02960	0.29	0.19
Lemon (IT)	BYI 02960	0.72	0.32
Mandarin (IT)	BYI 02960	0.90	0.45
Apple (IT)	BYI 02960	0.30	0.13
Pear (IT)	BYI 02960	0.47	0.22
Blueberry (IT)	BYI 02960	2.5	0.86
Grape (IT)	BYI 02960	2.3	0.58
Strawberry	BYI 02960	0.64	0.50
Prickly pear cactus (IT)	BYI 02960	0.13	0.12
Bulb onion	BYI 02960	0.06	0.026
Green/Welsh onion	BYI 02960	1.3	0.47
Head cabbage	BYI 02960	1.2	0.40
Flowerhead cabbage	BYI 02960	2.5	0.40
Cucumber	BYI 02960	0.25	0.13
Melon	BYI 02960	0.25	0.15
Summer squash	BYI 02960	0.11	0.064
Tomato (IT)	BYI 02960	0.88	0.17
Bell pepper (IT)	BYI 02960	0.35	0.11
Non-bell (chili) pepper (IT)	BYI 02960	0.58	0.25
Sweet corn	BYI 02960	0.05	0.01
Mustard green	BYI 02960	24	12
Lettuce (leafy)	BYI 02960	7.9	2.4
Lettuce (head)	BYI 02960	2.4	1.5
Spinach	BYI 02960	19	8.7
Beans with pods	BYI 02960	0.81	0.18
Peas with pods	BYI 02960	1.3	1.0
Beans, shelled	BYI 02960	0.12	0.028
Peas, shelled	BYI 02960	1.6	0.58
Beans, dry	BYI 02960	0.26	0.043
Peas, dry	BYI 02960	1.5	0.64
Soybean (IT)	BYI 02960	1.1	0.065
Carrot	BYI 02960	1.0	0.025
Potato (IT)	BYI 02960	0.057	0.01
Celery (IT)	BYI 02960	6.7	2.3
Barley, grain (IT)	DFA	1.2	0.34
Corn, grain (IT)	DFA	0.20	0.050
Sorghum, grain (IT)	DFA	0.14	0.053
Wheat, grain (IT)	DFA	2.27	0.52
Almond, nutmeat (IT)	BYI 02960	0.015	0.01
Pecan, nutmeat (IT)	BYI 02960	0.013	0.01
Cotton seed (IT)	BYI 02960	0.63	0.12
Peanuts (IT)	BYI 02960	0.034	0.01
Coffee (IT)	BYI 02960	0.60	0.14

Crop matrix	Marker compound	Residue level [mg/kg]	
Residues after a single foliar spray applications			
Hops (IT)	BYI 02960	4.7	2.7
Residues in rotational crops			
Barley hay	BYI 02960	26	4.8
Wheat forage	BYI 02960	15	0.52
Alfalfa forage	BYI 02960	8.7	3.6

Table 5.10-22: Transfer factors determined in the cattle and poultry feeding studies
(cf. 7.1/01, Table 7.1-11; cf. 7.2/01, Table 7.2-9)

Food item of animal origin	Transfer factor (total residue[1])
Cattle feeding study (dose level: 135 mg a.s./kg feed)	
Milk	0.007
Muscle	0.013
Fat	0.009
Liver	0.029
Kidney	0.036
Poultry feeding study (dose level: 1.5 mg a.s./kg feed)	
Eggs	0.038
Muscle	0.062
Fat	0.036
Liver	0.076

[1] total residue as defined for risk assessment and enforcement: sum of parent compound BYI 02960 and DFA

ESTIMATION OF THE CONSUMER EXPOSURE

(1) Occurrence of metabolites **BYI 02960-bromo/-chloro, BYI 02960-bromo-amino-furanone** and **BYI 02960-amino-furanone** in matrices of plant origin

Determination of the marker-to-metabolite ratio in the plant metabolism studies:

BYI 02960-bromo/-chloro:

Rotational crops, 1st rotation = representative for cereal feed items

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][1]			Ratio
	-bromo/-chloro	marker compound	label of selected study	marker comp. / metabolite
Wheat forage	0.003	0.365 (a.s.)	(F)	121.7
Wheat hay	0.021	0.601 (a.s.)	(P)	28.6

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.2.1/01, Table 2.2.2.1-2 (F) and 2.2.2.1/02, Table 2.2.2.1-9 (P)

Primary crops,
cotton intermediate = representative for hops after one foliar application
rice grain = representative for cereal grains after two foliar applications of BYI 02960

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][1]			Ratio
	-bromo/-chloro	marker compound	label of selected study	marker comp. / metabolite
Cotton intermediate	0.089	5.237 (a.s.)	(F)	58.8
Rice grain	0.009	0.24 (DFA)	(P)	26.7
Cotton gin trash	0.049	1.204 (a.s.)	(P)	24.6

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.1.4/01, Table 2.2.1.4-4 (cotton intermediate), cf. 2.2.1.5/02; Table 2.2.1.5-12 (rice grain) and cf. 2.2.1.4/02, Table 2.2.1.4-11 (cotton gin trash); for DFA values cf. 2.2.1.6/01

Estimation of metabolite levels in food items and feed stuff on basis of the marker concentration in the field residue trials:

Estimated residues for **BYI 02960-bomo/-chloro** = HR (marker) in field trial / ratio (marker / metabolite) in metabolism study

Crop matrix	Food /feed item	Ratio used	Residues in field studies [mg a.s. equiv./kg]		
			marker compound		estimated residue
Hops	food item	58.8	4.7	(a.s.)	0.080
Barley grain	food item	26.7	1.2	(DFA)	0.045
Corn grain	food item	26.7	0.20	(DFA)	0.007
Sorghum grain	food item	26.7	0.14	(DFA)	0.005
Wheat grain	food item	26.7	2.3	(DFA)	0.086
Wheat forage	feed item	121.7	15	(a.s.)	0.123
Alfalfa forage	feed item	121.7	8.7	(a.s.)	0.071
Barley hay	feed item	28.6	26	(a.s.)	0.909
Cotton gin trash	feed item	24.6	22	(a.s.)	0.894

Determination of the marker-to-metabolite ratio in the plant metabolism studies:

BYI 02960-bromo-amino-furanone:

Rotational crops, 1st rotation = representative for cereal feed items

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][1]			Ratio
	-bromo-amino-furanone	marker compound	label of selected study	marker comp. / metabolite
Wheat forage	0.016	0.365 (a.s.)	(F)	22.8
Wheat hay	0.033	0.672 (a.s.)	(F)	20.4

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.2.1/01, Table 2.2.2.1-2

Estimation of metabolite levels in food items and feed stuff on basis of the marker concentration in the field residue trials:

Estimated residues for **BYI 02960-bomo-amino-furanone** = HR (marker) in field trial / ratio (marker/metabolite) in metabolism study

Crop matrix	Food /feed item	Ratio used	Residues in field studies [mg a.s. equiv./kg]		
			marker compound		estimated residue
Wheat forage	feed item	22.8	15	(a.s.)	0.658
Alfalfa forage	feed item	22.8	8.7	(a.s.)	0.382
Barley hay	feed item	20.4	26	(a.s.)	1.275

Determination of the marker-to-metabolite ratio in the plant metabolism studies:

BYI 02960-amino-furanone:

Rotational crops, 1st rotation = representative for cereal feed items

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][1]			Ratio
	-amino-furanone	marker compound	label of selected study	marker comp. / metabolite
Wheat forage	0.015	0.365 (a.s.)	(F)	24.3
Wheat hay	0.076	0.672 (a.s.)	(F)	8.8

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.2.1/01, Table 2.2.2.1-2

Estimation of metabolite levels in food items and feed stuff on basis of the marker concentration in the field residue trials:

Estimated residues for **BYI 02960-amino-furanone** = HR (marker) in field trial / ratio (marker/metabolite) in metabolism study

Crop matrix	Food /feed item	Ratio used	Residues in field studies [mg a.s. equiv./kg]		
			marker compound		estimated residue
Wheat forage	feed item	24.3	15	(a.s.)	0.617
Alfalfa forage	feed item	24.3	8.7	(a.s.)	0.358
Barley hay	feed item	8.8	26	(a.s.)	2.955

Summary of estimated field residues of the metabolite group (1) consisting of metabolites **BYI 02960-bromo/-chloro, BYI 02960-bromo-amino-furanone** and **BYI 02960-amino-furanone** in food and feed items

Crop matrix	**Estimated residues in field studies [mg a.s. equiv./kg]**			
	-bromo /-chloro	-bromo-amino-furanone	-amino-furanone	sum metabolites
Hops	0.080	---	---	0.080
Barley grain	0.045	---	---	0.045
Corn grain	0.007	---	---	0.007
Sorghum grain	0.005	---	---	0.005
Wheat grain	0.075	---	---	0.075
Wheat forage	0.123	0.658	0.617	1.398
Alfalfa forage	0.071	0.382	0.368	0.817
Barley hay	0.909	1.275	2.955	5.139
Cotton gin trash	0.894	---	---	0.894

The transfer of the residues in food items of animal origin was estimated on basis of the transfer factors determined for the total residue in the cattle and poultry feeding studies.

Calculation of the dietary burden of livestock using the estimated field residues of the metabolite group (1):

- according to OECD dietary burden calculator

	Dietary burden of			
	dairy ruminant	**meat ruminants**	**poultry (broiler)**	**pigs**
Maximum dietary burden (mg/kg bw/d):	0.097	0.017	0.005	<0.004
Maximum dietary burden (mg/kg feed DM):	2.432	0.919	0.068	not relevant
Highest contributing commodity:	barley grain	barley grain	wheat grain	not relevant

Calculation of the consumer exposure to metabolite group (1) according to the intake model supported by JMPR, FAO panel

Input parameters:

- TTC of 1.5 µg/kg bw/day was set as ADI
- Estimated field residues of BYI 02960-amino-furanone, BYI 02960-bromo-amino-furanone and BYI 02960-bromo/-chloro were added and considered as a sum
- Residues in food items of animal origin were based on the dietary burden calculated according to OECD and the transfer factors estimated for the total residue (sum of parent BYI 02960 and DFA)

⇨ Residues used for calculation of IEDI calculation

Crop matrix	Residues [mg a.s.equiv./kg]
Hops	0.080
Barley grain	0.045
Corn grain	0.007
Sorghum grain	0.005
Wheat grain	0.075
Milk ruminant (dairy cattle)	0.017
Meat ruminant (beef cattle)	0.032
Fat ruminant (beef cattle)	0.022
Liver ruminant (beef cattle)	0.071
Kidney ruminant (beef cattle)	0.088
Eggs (poultry layer)	0.003
Meat poultry (poultry layer)	0.004
Fat poultry (poultry layer)	0.002
Liver poultry (poultry layer)	0.005

According to the IEDI calculations for the account of JMPR, the consumer exposure to the metabolite group (1), consisting of the three metabolites BYI 02960-bromo-chloro, BYI 02960-bromo-amino-furanone and BYI 02960-amino-furanone, is below the threshold of toxicological concern of 1.5 µg/kg bw/day. The theoretical maximum daily intake (IEDI) accounts in maximum for approx. 43% of the threshold level, i.e. for approx. 0.65 µg/kg bw/day. The most critical diet is the G02 diet with milk as largest contributor. Thus, it was clearly shown that the exposure to this metabolite group is so low that the probability of adverse health effects is negligible and that no further toxicological data are considered necessary.

(2) Occurrence of metabolite **BYI 02960-mercapto-lactic acid** in matrices of plant origin

Determination of the marker-to-metabolite ratio in the plant metabolism studies:

BYI 02960-mercapto-lactic acid:
Rotational crops, 1st rotation = representative for cereal feed items

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][1]			Ratio
	-mercapto-lactic acid	marker compound	label of selected study	marker comp. / metabolite
Wheat forage	0.013	0.365 (a.s.)	(F)	28.1
Wheat hay	0.008	0.672 (a.s.)	(F)	84.0

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.2.1/01, Table 2.2.2.1-2 (F)

Estimation of metabolite levels in food items and feed stuff on basis of the marker concentration in the field residue trials:

Estimated residues for **BYI 02960-mercapto-lactic acid** = HR (marker) in field trial / ratio (marker/metabolite) in metabolism study

Crop matrix	Food/feed item	Ratio used	Residues in field studies [mg a.s. equiv./kg]	
			marker compound	estimated residue
Wheat forage	feed item	28.1	15 (a.s.)	0.534
Alfalfa forage	feed item	28.1	35 (a.s.)	1.246
Barley hay	feed item	84.0	26 (a.s.)	0.310

BYI 02960-mercapto-lactatic acid was neither detected in wheat grains of the rotational crop studies nor in one of the target plant metabolism studies.

Calculation of the dietary burden of livestock using the estimated field residues of BYI 02960-mercapto-lactic acid:

- according to the OECD dietary burden calculator

	Dietary burden of			
	dairy ruminant	meat ruminants	poultry	pigs
Maximum dietary burden (mg/kg bw/d):	0.046	0.021	<0.004	<0.004
Maximum dietary burden (mg/kg feed DM):	1.157	0.053	not relevant	not relevant
Highest contributing commodity:	alfalfa forage wheat forage	barley hay	not relevant	not relevant

Calculation of the consumer exposure to BYI 02960-mercapto-lactic acid according to the intake model supported by JMPR, FAO panel

Input parameters:

- TTC of 1.5 µg/kg bw/day was set as ADI
- Estimated field residues of BYI 02960-mercapto-lactic acid were used for livestock dietary burden calculation
- Residues in food items of animal origin were estimated on the dietary burden calculated according to OECD and the transfer factors estimated for the total residue

⇨ Residues used for calculation of IEDI calculation

Crop matrix	Residues [mg a.s.equiv./kg]
Milk ruminant (dairy cattle)	0.008
Meat ruminant (beef cattle)	0.015
Fat ruminant (beef cattle)	0.010
Liver ruminant (beef cattle)	0.034
Kidney ruminant(beef cattle)	0.042

According to the IEDI calculations for the account of JMPR, the consumer exposure to metabolite BYI 02960-mercapto-lactic acid is below the threshold of toxicological concern of 1.5 µg/kg bw/day. The theoretical maximum daily intake (IEDI) accounts in maximum for approx. 0.6% of the threshold level, and is thus negligible.

Since no food item of plant origin showed the metabolite, it can be concluded that the chronic and acute exposure to the metabolite is so low that the probability of adverse health effects is negligible and no further data are considered necessary.

(3) Occurrence of metabolite **BYI 02960-acetic acid and its conjugate BYI 02960-acetic acid-glyc** in matrices of plant origin

Determination of the marker-to-metabolite ratio in the plant metabolism studies:

BYI 02960-acetic acid:

Rotational crops, 1st rotation = representative for cereal feed items

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][1]				Ratio
	-acetic acid	marker compound		label of selected study	marker comp. / metabolite
Wheat forage	0.013	0.365	(a.s.)	(F)	28.1
Wheat hay	0.047	0.676	(a.s.)	(P)	14.4

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.2.1/01, Table 2.2.2.1-2 (F), cf. 2.2.2.1/02, Table 2.2.2.1-9 (P)

Primary crops,
apple = representative for all fruiting crops, leafy crops, legume vegetables and celery after two foliar applications
cotton interm. = representative for hops after one foliar application
cotton seed = representative for almond and pecan, peanut, soybean, cotton and coffee after two foliar applications
rice grain = representative for cereal grains after two foliar applications

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][2]				Ratio
	-acetic acid	marker compound		label of selected study	marker comp. / metabolite
Apple	0.009	0.946	(a.s.)	(F)	105.1
Cotton intermediate	3.082[1]	5.237	(a.s.)	(F)	1.7
Cotton seed	0.003	0.016	(a.s.)	(P)	5.3
Rice grain	0.048	0.24	(DFA)	(P)	5.0
Cotton gin trash	0.577[1]	1.505	(a.s.)	(F)	2.6

(F) = furanone label (P) = pyridinylmethyl label

[1] co-elution with BYI 02960-OH-glyc (overestimation of metabolite concentration)

[2] cf. 2.2.1.3, Table 2.2.1.3-11 (apple), 2.2.1.4/01, Table 2.2.1.4-6 (cotton intermediate and gin trash) and Table 2.2.1.4-10 (cotton seeds); cf. 2.2.1.5/03, Table 2.2.1.5-12 (rice); for DFA data see 2.2.1.6/01

Estimation of metabolite levels in food items and feed stuff on basis of the marker concentration in the field residue trials:

Estimated residues for **BYI 02960-acetic acid** = HR or STMR (marker) in field trial / ratio (marker/metabolite) in metabolism study

Crop matrix	Food /feed item	Ratio used	Residues in field studies [mg a.s. equiv./kg]				
			marker compound			estimated residue	
			HR	STMR		HR	STMR
Orange	food item	105.1	2.1	0.31	(a.s.)	0.020	0.003
Grapefruit	food item	105.1	0.29	0.19	(a.s.)	0.003	0.002
Lemon	food item	105.1	0.72	0.32	(a.s.)	0.007	0.003
Mandarin	food item	105.1	0.90	0.45	(a.s.)	0.009	0.004
Apple	food item	105.1	0.30	0.13	(a.s.)	0.003	0.001
Pear	food item	105.1	0.47	0.22	(a.s.)	0.004	0.002
Blueberry	food item	105.1	2.5	0.86	(a.s.)	0.024	0.008
Grape	food item	105.1	2.3	0.58	(a.s.)	0.022	0.006
Strawberry	food item	105.1	0.64	0.50	(a.s.)	0.006	0.005
Prickly pear cactus	food item	105.1	0.15	0.12	(a.s.)	0.001	0.001
Cucumber	food item	105.1	0.25	0.13	(a.s.)	0.002	0.001
Melon	food item	105.1	0.25	0.15	(a.s.)	0.002	0.001
Summer squash	food item	105.1	0.11	0.064	(a.s.)	0.001	0.001
Tomato	food item	105.1	0.88	0.17	(a.s.)	0.008	0.002
Bell pepper	food item	105.1	0.35	0.11	(a.s.)	0.003	0.001
Non-bell (chili) pepper	food item	105.1	0.58	0.25	(a.s.)	0.006	0.002
Sweet corn	food item	105.1	0.05	0.01	(a.s.)	0.000	0.000
Head cabbage	food item	105.1	1.2	0.40	(a.s.)	0.011	0.004
Flowerhead cabbage	food item	105.1	2.5	0.40	(a.s.)	0.024	0.004
Mustard green	food item	105.1	24	12	(a.s.)	0.228	0.114
Lettuce	food item	105.1	7.9	2.4	(a.s.)	0.075	0.023
Spinach	food item	105.1	19	8.7	(a.s.)	0.181	0.083
Beans with pods	food item	105.1	0.81	0.18	(a.s.)	0.008	0.002
Peas with pods	food item	105.1	1.3	1.0	(a.s.)	0.012	0.010
Beans, shelled	food item	105.1	0.12	0.028	(a.s.)	0.001	0.000
Peas, shelled	food item	105.1	1.6	0.58	(a.s.)	0.015	0.006
Celery	food item	105.1	6.7	2.3	(a.s.)	0.064	0.022
Hops	food item	1.7	4.7	2.7	(a.s.)	2.765	1.588
Almond	food item	5.3	0.015	0.01	(a.s.)	0.003	0.002
Pecan	food item	5.3	0.013	0.01	(a.s.)	0.002	0.002
Peanut	food item	5.3	0.034	0.01	(a.s.)	0.006	0.002
Soybean	food item	5.3	1.1	0.065	(a.s.)	0.208	0.012
Cotton seed	food item	5.3	0.63	0.12	(a.s.)	0.119	0.023
Coffee	food item	5.3	0.60	0.14	(a.s.)	0.113	0.026
Barley grain	food item	5.0	1.2	0.34	(DFA)	0.240	0.068
Corn grain	food item	5.0	0.17	0.050	(DFA)	0.034	0.010
Sorghum grain	food item	5.0	0.20	0.053	(DFA)	0.040	0.011
Wheat grain	food item	5.0	2.27	0.52	(DFA)	0.454	0.104
Wheat forage	feed item	28.1	15	0.52	(a.s.)	0.534	0.019
Alfalfa forage	feed item	28.1	8.7	3.6	(a.s.)	0.310	0.128

Crop matrix	Food /feed item	Ratio used	Residues in field studies [mg a.s. equiv./kg]				
			marker compound			estimated residue	
			HR	STMR		HR	STMR
Barley hay	feed item	14.4	26	4.8	(a.s.)	1.806	0.333
Cotton gin trash	feed item	2.6	22	10	(a.s.)	8.462	3.846
Apple, wet pomace*	feed item	89.9	0.44	0.21	(a.s.)	0.005	0.002

Determination of the marker-to-metabolite ratio in the plant metabolism studies:

BYI 02960-acetic acid-glyc:

Rotational crops, 1st rotation = representative for cereal feed items

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][1]				Ratio
	-acetic acid-glyc	marker compound		label of selected study	marker comp. / metabolite
Wheat forage	0.009	0.640	(a.s.)	(P)	71.1

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.2.1/02, Table 2.2.2.1-9

Primary crops,

apple = representative for all fruiting crops, leafy crops, legume vegetables and celery after two foliar applications

cotton interm. = representative for hops after one foliar application

rice grain = representative for cereal grains after two foliar applications

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][2]				Ratio
	-acetic acid-glyc	marker compound		label of selected study	marker comp. / metabolite
Apple	0.007	0.946	(a.s.)	(F)	135.1
Cotton intermediate	1.068	5.237	(a.s.)	(F)	4.9
Rice grain	0.003	0.24	(DFA)	(P)	80.0
Cotton gin trash	0.087	1.247	(a.s.)	(P)	14.3

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.1.3, Table 2.2.1.3-11 (apple), 2.2.1.4/01, Table 2.2.1.4-6 (cotton intermediate and gin trash) and Table 2.2.1.4-10 (cotton seeds); cf. 2.2.1.5/02, Table 2.2.1.5-12 (rice); for DFA-data see 2.2.1.6/01

Estimation of metabolite levels in food items and feed stuff on basis of the marker concentration in the field residue trials:

Estimated residues for **BYI 02960-acetic acid-glyc** = HR (marker) in field trial / ratio (marker/metabolite) in metabolism study

Crop matrix	Food /feed item	Ratio used	Residues in field studies [mg a.s. equiv./kg]				
			marker compound			estimated residue	
			HR	STMR		HR	STMR
Orange	food item	135.1	2.1	0.31	(a.s.)	0.016	0.002
Grapefruit	food item	135.1	0.29	0.19	(a.s.)	0.002	0.001
Lemon	food item	135.1	0.72	0.32	(a.s.)	0.005	0.002
Mandarin	food item	135.1	0.90	0.45	(a.s.)	0.007	0.003
Apple	food item	135.1	0.30	0.15	(a.s.)	0.002	0.001
Pear	food item	135.1	0.47	0.22	(a.s.)	0.003	0.002
Blueberry	food item	135.1	2.5	0.86	(a.s.)	0.019	0.006
Grape	food item	135.1	2.3	0.58	(a.s.)	0.017	0.004
Strawberry	food item	135.1	0.64	0.50	(a.s.)	0.005	0.004
Prickly pear cactus	food item	135.1	0.15	0.12	(a.s.)	0.001	0.001
Cucumber	food item	135.1	0.25	0.13	(a.s.)	0.002	0.001
Melon	food item	135.1	0.25	0.15	(a.s.)	0.002	0.001
Summer squash	food item	135.1	0.11	0.064	(a.s.)	0.001	0.000
Tomato	food item	135.1	0.88	0.19	(a.s.)	0.007	0.001
Bell pepper	food item	135.1	0.35	0.11	(a.s.)	0.003	0.001
Non-bell (chili) pepper	food item	135.1	0.58	0.25	(a.s.)	0.004	0.002
Sweet corn	food item	135.1	0.05	0.01	(a.s.)	0.000	0.000
Head cabbage	food item	135.1	1.2	0.40	(a.s.)	0.009	0.003
Flowerhead cabbage	food item	135.1	2.5	0.40	(a.s.)	0.019	0.003
Mustard green	food item	135.1	24	12	(a.s.)	0.178	0.089
Lettuce	food item	135.1	7.9	2.4	(a.s.)	0.058	0.018
Spinach	food item	135.1	19	8.7	(a.s.)	0.141	0.064
Beans with pods	food item	135.1	0.81	0.18	(a.s.)	0.006	0.001
Peas with pods	food item	135.1	1.3	1.0	(a.s.)	0.010	0.007
Beans, shelled	food item	135.1	0.12	0.028	(a.s.)	0.001	0.000
Peas, shelled	food item	135.1	1.6	0.58	(a.s.)	0.012	0.004
Celery	food item	135.1	6.7	2.3	(a.s.)	0.050	0.017
Hops	food item	74.9	4.7	2.7	(a.s.)	0.959	0.551
Barley grain	food item	80.0	1.2	0.34	(a.s.)	0.015	0.004
Corn grain	food item	80.0	0.20	0.050	(a.s.)	0.003	0.001
Sorghum grain	food item	80.0	0.14	0.053	(a.s.)	0.002	0.001
Wheat grain	food item	80.0	2.27	0.52	(a.s.)	0.028	0.007
Wheat forage	feed item	71.1	15	0.52	(a.s.)	0.211	0.007
Alfalfa forage	feed item	71.1	8.7	3.6	(a.s.)	0.122	0.051
Cotton gin trash	feed item	14.3	22	10	(a.s.)	1.538	0.699
Apple wet pomace*	feed item	135.1	0.44	0.21	(a.s.)	0.005	0.002

* median processing factor for apple: 1.45

Summary of estimated field residues of the metabolite group (3) consisting of metabolites **BYI 02960-acetic acid** and **BYI 02960-acetic acid-glyc** in food and feed items

Crop matrix	**Estimated residues in field studies [mg a.s. equiv./kg]**					
	-acetic acid		-acetic acid-glyc		sum metabolites	
	HR	STMR	HR	STMR	HR	STMR
Orange	0.020	0.003	0.016	0.002	0.036	0.005
Grapefruit	0.003	0.002	0.002	0.001	0.005	0.003
Lemon	0.007	0.003	0.005	0.002	0.012	0.005
Mandarin	0.009	0.004	0.007	0.003	0.015	0.008
Apple	0.003	0.001	0.002	0.001	0.005	0.003
Pear	0.004	0.002	0.003	0.002	0.008	0.004
Grape	0.024	0.008	0.019	0.006	0.042	0.015
Blueberry	0.022	0.006	0.017	0.004	0.039	0.010
Strawberry	0.006	0.005	0.005	0.004	0.011	0.008
Prickly pear cactus	0.001	0.001	0.001	0.001	0.003	0.002
Cucumber	0.002	0.001	0.002	0.001	0.004	0.002
Melon	0.002	0.001	0.002	0.001	0.004	0.003
Summer squash	0.001	0.001	0.001	0.000	0.002	0.001
Tomato	0.008	0.002	0.007	0.001	0.015	0.003
Bell pepper	0.003	0.001	0.003	0.001	0.006	0.002
Non-bell (chili) pepper	0.006	0.002	0.004	0.002	0.010	0.004
Sweet corn	0.000	0.000	0.000	0.000	0.001	0.000
Head cabbage	0.011	0.004	0.009	0.003	0.020	0.007
Flowerhead cabbage	0.024	0.004	0.019	0.003	0.042	0.007
Mustard green	0.228	0.114	0.178	0.089	0.406	0.203
Lettuce	0.075	0.023	0.058	0.018	0.134	0.041
Spinach	0.181	0.083	0.141	0.064	0.321	0.147
Beans with pods	0.008	0.002	0.006	0.001	0.014	0.003
Peas with pods	0.012	0.010	0.010	0.007	0.022	0.017
Beans, shelled	0.001	0.000	0.001	0.000	0.002	0.000
Peas, shelled	0.015	0.006	0.012	0.004	0.027	0.010
Celery	0.064	0.022	0.050	0.017	0.113	0.039
Hops	2.765	1.588	0.959	0.551	3.724	2.139
Almond	0.003	0.002	---	---	0.003	0.002
Pecan	0.002	0.002	---	---	0.002	0.002
Peanut	0.006	0.002	---	---	0.006	0.002
Soybean	0.208	0.012	---	---	0.208	0.012
Cotton seed	0.119	0.023	---	---	0.119	0.023
Coffee	0.113	0.026	---	---	0.113	0.026
Barley grain	0.240	0.068	0.015	0.004	0.255	0.072
Corn grain	0.034	0.010	0.003	0.001	0.037	0.011
Sorghum grain	0.040	0.011	0.002	0.001	0.042	0.011
Wheat grain	0.454	0.104	0.028	0.007	0.482	0.111
Wheat forage	0.534	0.019	0.211	0.007	0.745	0.026
Alfalfa forage	0.310	0.128	0.122	0.051	0.432	0.179
Barley hay	1.806	0.333	---	---	1.806	0.333
Cotton gin trash	8.462	3.846	1.538	0.699	10.000	4.545
Apple, wet pomace	0.005	0.002	0.005	0.002	0.010	0.005

Calculation of the dietary burden of livestock using the estimated field residues of BYI 02960-acetic acid and its conjugate BYI 02960-acetic acid-glyc:

- according to the OECD dietary burden

	Dietary burden of			
	dairy ruminant	meat ruminants	poultry (broiler)	pigs
Maximum dietary burden (mg/kg bw/d):	0.045	0.010	0.008	<0.004
Maximum dietary burden (mg/kg feed DM):	1.115	0.526	0.104	not relevant
Highest contributing commodity:	barley grain	barley grain	wheat grain	not relevant

Calculation of the consumer exposure to metabolite group (3) according to the intake model supported by JMPR, FAO panel:

Input parameters:

- TTC of 1.5 µg/kg bw/day was set as ADI.
- Estimated field residues of BYI 02960-acetic acid and BYI 02960-acetic acid-glyc were added and considered as a sum.
- Residues in food items of animal origin were based on the dietary burden calculated according to OECD (worst case for meat ruminants and poultry) and the transfer factors estimated for the total residue (sum of BYI 02960 and DFA).

⇨ Residues used for calculation:

Crop matrix	Residues [mg a.s.equiv./kg]	
	HR	STMR
Orange	0.036	0.005
Grapefruit	0.005	0.003
Lemon	0.012	0.005
Mandarin	0.015	0.008
Apple	0.005	0.003
Pear	0.008	0.004
Grape	0.042	0.015
Blueberry	0.039	0.010
Strawberry	0.011	0.008
Prickly pear cactus	0.003	0.002
Cucumber	0.004	0.002
Melon	0.004	0.003
Summer squash	0.002	0.001
Tomato	0.015	0.003
Bell pepper	0.006	0.002
Non-bell (chili) pepper	0.010	0.004
Sweet corn	0.001	0.000
Head cabbage	0.020	0.007
Flowerhead cabbage	0.042	0.007
Mustard green	0.406	0.203

Crop matrix	Residues [mg a.s.equiv./kg]	
	HR	STMR
Lettuce	0.134	0.041
Spinach	0.321	0.147
Beans with pods	0.014	0.003
Peas with pods	0.022	0.017
Beans, shelled	0.002	0.000
Peas, shelled	0.027	0.010
Celery	0.113	0.039
Hops	3.724	2.139
Almond	0.003	0.002
Pecan	0.002	0.002
Peanut	0.006	0.002
Soybean	0.208	0.042
Cotton seed	0.119	0.023
Coffee	0.113	0.026
Barley grain	0.255	0.072
Corn grain	0.037	0.011
Sorghum grain	0.042	0.011
Wheat grain	0.482	0.111
Milk (dairy cattle)	0.008	0.008
Meat ruminant (beef cattle)	0.014	0.014
Fat ruminant (beef cattle)	0.010	0.010
Liver ruminant (beef cattle)	0.032	0.032
Kidney ruminant (beef cattle)	0.040	0.040
Eggs (poultry layer)	0.004	0.004
Meat poultry (poultry layer)	0.006	0.006
Fat poultry (poultry layer)	0.003	0.003
Liver poultry (poultry layer)	0.008	0.008

According to the IEDI calculations for the account of JMPR, the consumer exposure to the metabolite group (3), consisting of the two metabolites BYI 02960-acetic acid and BYI 02960-acetic acid-glyc, is above the threshold of toxicological concern of 1.5 µg/kg bw/day for chronic exposure when using the HR values but below the threshold of 5 µg/kg for acute exposure. Since the first chronic estimations were done with highest residues (HR), the calculation has to be refined by using median residues (STMR). The calculation was done accordingly and the international estimated daily intake (IEDI) accounts now in maximum for approx. 63% of the threshold level, i.e. for approx. 0.95 µg/kg bw/day in the most critical diet (G06). The largest contributor is wheat. It was clearly shown that the exposure to this metabolite group is so low that the probability of adverse health effects is negligible and that no further data are considered necessary.

(4) Occurrence of metabolites **BYI 02960-glyoxylic acid**, **BYI 02960-AMCP-difluoroethanamine**, and **BYI 02960-N-formyl-** and **-N-acetyl-AMCP-difluoroethanamine** in matrices of plant origin

Determination of the marker-to-metabolite ratio in the plant metabolism studies:

BYI 02960-glyoxylic acid:

Rotational crops, 1st rotation = representative for cereals, leafy crops and root crops

Crop matrix	**Residues in metabolism studies [mg a.s. equiv./kg][1]**				**Ratio**
	-glyoxylic acid	marker compound		label of selected study	marker comp. / metabolite
Wheat forage	0.124	0.365	(a.s.)	(F)	2.9
Wheat hay	0.227	0.676	(a.s.)	(P)	3.0

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.2.1/01, Table 2.2.2.1-2

Primary crops,
cotton interm. = representative for hops after one foliar application
rice grain = representative for cereal grains after two foliar applications of BYI 02960

Crop matrix	**Residues in metabolism studies [mg a.s. equiv./kg][1]**				**Ratio**
	- glyoxylic acid	marker compound		label of selected study	marker comp. / metabolite
Cotton intermediate	0.209	5.221	(a.s.)	(P)	25.0
Rice grain	0.003	0.24	(DFA)	(P)	80.0

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.1.4/02, Table 2.2.1.4-10 (cotton intermediate) and Table 2.2.1.4-10, cf. 2.2.1.5/02, Table 2.2.1.5-12 (rice); for DFA data see 2.2.1.6/01

Estimation of metabolite levels in food items and feed stuff on basis of the marker concentration in the field residue trials:

Estimated residues for **BYI 02960-glyoxylic acid** = HR (marker) in field trial / ratio (marker/metabolite) in metabolism study

Crop matrix	**Food /feed item**	**Ratio used**	**Residues in field studies [mg a.s. equiv./kg]**		
			marker comp.(HR)		estimated residue
Hops	food item	25.0	4.63	(a.s.)	0.185
Barley grain	food item	80.0	0.086	(DFA)	0.001
Corn grain	food item	80.0	0.171	(DFA)	0.002
Sorghum grain	food item	80.0	0.051	(DFA)	0.001
Wheat grain	food item	80.0	0.482[1]	(DFA)	0.006
Barley hay	feed item	3.0	26	(a.s.)	8.7
Wheat forage	feed item	2.9	15	(a.s.)	5.2
Alfalfa forage	feed item	2.9	8.7	(a.s.)	3.0

Determination of the marker-to-metabolite ratio in the plant metabolism studies:

BYI 02960-AMCP-difluoroethanamine:

Primary crops,
apple = representative for all fruiting crops, leafy crops, legume vegetables and celery after two foliar applications

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][1]			Ratio
	-AMCP-difluoroethanamine	marker compound	label of selected study	marker comp.: metabolite
Apple	0.085	1.652 (a.s.)	(P)	19.4

(F) = furanone label (P) = pyridinylmethyl label

[1] cf. 2.2.1.3/02, Table 2.2.1.3-9 (apple)

Estimation of metabolite levels in food items and feed stuff on basis of the marker concentration in the field residue trials:

Estimated residues for **BYI 02960-AMCP-difluoroethanamine** = HR (marker) in field trial / ratio (marker/metabolite) in metabolism study

Crop matrix	Food /feed item	Ratio used	Residues in field studies [mg a.s. equiv./kg]		
			marker compound		estimated residue
Orange (IT)	food item	19.4	2.1	(a.s.)	0.108
Grapefruit (IT)	food item	19.4	0.29	(a.s.)	0.015
Lemon (IT)	food item	19.4	0.72	(a.s.)	0.037
Mandarin (IT)	food item	19.4	0.90	(a.s.)	0.046
Apple (IT)	food item	19.4	0.30	(a.s.)	0.015
Pear (IT)	food item	19.4	0.47	(a.s.)	0.024
Blueberry (IT)	food item	19.4	2.5	(a.s.)	0.129
Grape (IT)	food item	19.4	2.3	(a.s.)	0.119
Strawberry	food item	19.4	0.64	(a.s.)	0.033
Prickly pear cactus (IT)	food item	19.4	0.15	(a.s.)	0.008
Cucumber	food item	19.4	0.25	(a.s.)	0.013
Melon	food item	19.4	0.25	(a.s.)	0.013
Summer squash	food item	19.4	0.11	(a.s.)	0.006
Tomato	food item	19.4	0.88	(a.s.)	0.045
Bell pepper	food item	19.4	0.35	(a.s.)	0.018
Non-bell (chili) pepper	food item	19.4	0.58	(a.s.)	0.030
Sweet corn	food item	19.4	0.05	(a.s.)	0.003
Head cabbage	food item	19.4	1.2	(a.s.)	0.062
Flowerhead cabbage	food item	19.4	2.5	(a.s.)	0.129
Mustard green	food item	19.4	24	(a.s.)	1.237
Lettuce	food item	19.4	7.9	(a.s.)	0.407
Spinach	food item	19.4	19	(a.s.)	0.979
Beans with pods	food item	19.4	0.81	(a.s.)	0.042
Peas with pods	food item	19.4	1.3	(a.s.)	0.067
Beans, shelled	food item	19.4	0.12	(a.s.)	0.006
Peas, shelled	food item	19.4	1.6	(a.s.)	0.082
Celery	food item	19.4	0.25	(a.s.)	0.013

Metabolites **BYI 02960-N-formyl- /BYI 02960-N-acetyl-AMCP-difluoroethanamine** and were only detected in Swiss chard and turnips in the confined rotational crop studies – but in none of the metabolism studies on primary crops. Therefore the metabolite levels were only estimated the root, tuber, and bulb vegetables, as well as in pulses grown in rotation. The field rotational studies – used to estimate the metabolite concentrations - were conducted in Europe according to the EU GAP, which is less critical. To consider this fact, the residues of the marker compounds were extrapolated according to the North American use pattern and in addition a soil accumulation factor of 1.2 was applied.

Determination of the marker-to-metabolite ratio in the plant metabolism studies:

BYI 02960-N-formyl- /BYI 02960-N-acetyl-AMCP-difluoroethanamine
Rotational crops, 1st rotation = representative for leafy and root crops

Crop matrix	Residues in metabolism studies [mg a.s. equiv./kg][1]				Ratio
	-N-formyl- / -N-acetyl-AMCP-difluoroethanamine	marker compound		label of selected study	marker comp. / metabolite
Swiss chard	0.043	0.48	(DFA)	(P)	11.2
Turnip roots	0.003	0.06	(DFA)	(P)	20.0
Turnip tops	0.030	0.24	(DFA)	(P)	8.0

(F) = furanone label (P) = pyridinylmethyl label
[1] cf. 2.2.2.2, Table 2.2.2.1-12 (P)

Estimation of metabolite levels in food items and feed stuff on basis of the marker concentration in the field residue trials:

Estimated residues for **BYI 02960-N-formyl- /BYI 02960-N-acetyl-AMCP-difluoroethanamine** = HR (marker) in field trial / ratio (marker/metabolite) in metabolism study

Crop matrix	Food / feed item	Ratio used	Residues in field studies [mg a.s. equiv./kg]		
			marker compound		estimated residue
Leafy crops (RC)	food item	11.2	0.30	(DFA)	0.027
Leek (RC)	food item	11.2	0.45	(DFA)	0.040
Green bean (RC)	food item	11.2	2.2	(DFA)	0.196
Carrots (RC)	food item	20.0	0.15	(DFA)	0.008
Field pea, dried seed (RC)	food item	20.0	4.5	(DFA)	0.225
Onion (RC)	food item	20.0	0.31	(DFA)	0.016
Turnip roots (RC)	food item	20.0	0.30	(DFA)	0.015
Turnip tops (RC)	feed item	8.0	0.49	(DFA)	0.061

(RC) residues result from EU field rotational crops were extrapolated to fit the North American GAP

Summary of estimated field residues of the metabolite group (4) consisting of metabolites **BYI 02960-glyoxylic acid**, **BYI 02960-AMCP-difluoroethanamine**, and **BYI 02960-N-formyl-** and **-N-acetyl-AMCP-difluoroethanamine** in food and feed items

Crop matrix		**Estimated residues in field studies [mg a.s. equiv./kg]**		
	-glyoxylic acid	-AMCP-difluoroethanamine	-N-formyl-/ -N-acetyl-AMCP-difluoroethanamine	sum metabolites
Orange (IT)	---	0.108	---	0.108
Grapefruit (IT)	---	0.015	---	0.015
Lemon (IT)	---	0.037	---	0.037
Mandarin (IT)	---	0.046	---	0.046
Apple (IT)	---	0.015	---	0.015
Pear (IT)	---	0.024	---	0.024
Blueberry (IT)	---	0.129	---	0.129
Grape (IT)	---	0.119	---	0.119
Strawberry	---	0.033	---	0.033
Prickly pear cactus (IT)	---	0.008	---	0.008
Cucumber	---	0.013	---	0.013
Melon	---	0.013	---	0.013
Summer squash	---	0.006	---	0.006
Tomato	---	0.045	---	0.045
Bell pepper	---	0.018	---	0.018
Non-bell (chili) pepper	---	0.030	---	0.030
Sweet corn	---	0.003	---	0.003
Head cabbage	---	0.062	---	0.062
Flowerhead cabbage	---	0.129	---	0.129
Mustard green	---	1.237	0.027	1.264
Lettuce	---	0.407	0.027	0.434
Spinach	---	0.979	0.027	1.006
Beans with pods	---	0.042	0.196	0.238
Peas with pods	---	0.067	---	0.067
Beans, shelled	---	0.006	---	0.006
Peas, shelled	---	0.082	---	0.082
Celery (IT)	---	0.013	---	0.013
Hops	0.185	---	---	0.185
Barley grain	0.001	---	---	0.001
Corn grain	0.002	---	---	0.002
Sorghum grain	0.001	---	---	0.001
Wheat grain	0.006	---	---	0.006
Barley hay	8.7	---	---	8.7
Wheat forage	5.2	---	---	5.2
Alfalfa forage	3.0	---	---	3.0
Leek (RC)[2]	---	---	0.040	0.040
Carrots (RC)[2]	---	---	0.008	0.008
Field pea, dried seed (RC)[2]	---	---	0.225	0.225
Onion (RC)[2]	---	---	0.016	0.016
Turnip roots (RC)[2]	---	---	0.015	0.015
Turnip tops (RC)[2]	---	---	0.061	0.061

The residue levels of the metabolites BYI 02960-glyoxylic acid, BYI 02960-AMCP-difluoroethanamine, and BYI 02960-N-formyl /BYI 02960-N-acetyl-AMCP-difluoroethanamine in feed items were used to calculate the animal burden. The transfer of the residues in food items of animal origin was estimated on basis of the transfer factors determined for DFA in the cattle and poultry feeding studies.

Calculation of the dietary burden of livestock using the estimated field residues of the metabolite group (4):

- according to the OECD dietary burden calculator

	Dietary burden of			
	dairy ruminant	**meat ruminants**	**poultry**	**pigs**
Maximum dietary burden (mg/kg bw/d):	0.264	0.0027	<0.004	<0.004
Maximum dietary burden (mg/kg feed DM):	6.589	1.486	not relevant	not relevant
Highest contributing commodity:	barley hay, wheat forage	corn grain	not relevant	not relevant

Calculation of the consumer exposure to metabolite group (4) according to the intake model supported by JMPR, FAO panel

Input parameters:

- TTC of 1.5 µg/kg bw/day was set as ADI.
- Estimated field residues of BYI 02960-glyoxylic acid, BYI 02960-AMCP-difluoroethanamine, and BYI 02960-N-formyl /BYI 02960-N-acetyl-AMCP-difluoroethanamine were added and considered as a sum.
- Residues in food items of animal origin were based on the dietary burden calculated according to OECD (worst case for meat ruminants and poultry) and the transfer factors estimated for metabolite DFA (worst case transfer)

⇨ Residues used for calculation:

Crop matrix	**Residues [mg a.s.equiv./kg]**
Orange (IT)	0.108
Grapefruit (IT)	0.015
Lemon (IT)	0.037
Mandarin (IT)	0.046
Apple (IT)	0.015
Pear (IT)	0.024
Blueberry (IT)	0.129
Grape (IT)	0.119
Strawberry	0.033
Prickly pear cactus (IT)	0.008
Cucumber	0.013
Melon	0.013
Summer squash	0.006
Tomato	0.045

Crop matrix	Residues [mg a.s.equiv./kg]
Bell pepper	0.018
Non-bell (chili) pepper	0.030
Sweet corn	0.003
Head cabbage	0.062
Flowerhead cabbage	0.129
Mustard green	1.264
Lettuce	0.434
Spinach	1.006
Beans with pods	0.238
Peas with pods	0.067
Beans, shelled	0.006
Peas, shelled	0.082
Celery (IT)	0.013
Hops	0.185
Barley grain	0.001
Corn grain	0.002
Sorghum grain	0.001
Wheat grain	0.006
Leek (RC)[2]	0.040
Carrots (RC)[2]	0.008
Field pea, dried seed (RC)[2]	0.225
Onion (RC)[2]	0.016
Turnip roots (RC)[2]	0.015
Turnip tops (RC)[2]	0.061
Milk (dairy cattle)	0.046
Meat ruminant (beef cattle)	0.086
Fat ruminant (beef cattle)	0.059
Liver ruminant (beef cattle)	0.190
Kidney ruminant (beef cattle)	0.240

According to the IEDI calculations for the account of JMPR, the consumer exposure to the metabolite group (4), consisting of the four metabolites BYI 02960-glyoxylic acid, BYI 02960-AMCP-difluoroethanamine, and BYI 02960-N-formyl- and -N-acetyl-AMCP-difluoroethanamine, is below the threshold of toxicological concern of 1.5 µg/kg bw/day, even when calculating with the highest field residues (HR) as worst-case estimation. The international estimated daily intake (IEDI) accounts in maximum for approx. 91% of the threshold level, i.e. for approx. 1.4 µg/kg bw/day in the most critical diet (G07 diet). Thus, it was shown that the exposure to this metabolite group is so low that the probability of adverse health effects is negligible and that no further data are considered necessary.

According to these estimations, the residue concentration of each metabolite is below the threshold of toxicological concern of 1.5 µg/kg bw. The threshold value was never exceeded even when grouping the metabolites and even when calculating with the highest residues instead of the median residues (which would be more appropriate for chronic exposure estimations), except for metabolite group (3) comprising BYI 02960-acetic acid and BYI 02960-acetic acid-glyc STMR values had to be used. Thus

the probability of adverse health effects due to the dietary exposure to these metabolites is negligible and no further tox testing is considered necessary.

(5) Metabolites with additional toxicity testing

Due to various considerations, additional toxicity studies were initiated for the metabolite difluoroacetic acid (a major soil metabolite with the potential, according to FOCUS groundwater modelling, to leach into shallow groundwater at concentrations of > 0.75 mg/L) and metabolite BYI 02960-difluoroethyl-amino-furanone (a proposed constituent of the residue definition for data collection in target and rotational crops; it was observed in the rat ADME study at levels less than 5% of the administered dose). Additional toxicity data are also available for metabolites BYI 02960-CHMP and 6-CNA. They are common metabolites with the active substance acetamiprid and several toxicological studies had been conducted for the submission of this insecticide. As described in detail in paragraph 5.8, none of these metabolites are acutely toxic or exhibit a genotoxic potential. The metabolites tested after subchronic (difluoroacetic acid, BYI 02960-CHMP) or subacute (BYI 02960-difluoroethyl-amino-furanone) administration to the rat were less toxic than BYI 02960 and are thus covered by the endpoints derived for the parent compound BYI 02960.

(6) Metabolites of no toxicological concern

For the natural compounds glucose, lactose and fatty acids no estimation of residue levels was considered necessary since they are of no toxicological concern.

Conclusions

A health risk due to the dietary exposure to plant and livestock metabolites of BYI 02960 can be excluded on basis of the data presented. All metabolites detected show either a consumer exposure below the agreed threshold level of toxicological concern (<1.5 µg/kg bw), or it has been shown that the metabolites are covered by the endpoints derived for the parent compound BYI 02960.
The aim of the estimations was to exclude a chronic risk for the consumer, however since all calculations were done on the basis of highest residue values (HR), also an acute risk can be excluded. The threshold value for the acute risk is 5.0 µg/kg bw/day for a compound assigned to Cramer Class III and thus all metabolites are covered by the calculations presented.

An overall evaluation of the plant and livestock metabolites is summarized in Table 5.10-23.

Table 5.10-23 Evaluation of plant and livestock metabolites

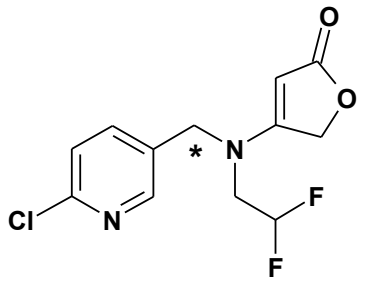
BYI 02960 metabolites of/with		
no toxicity concern	**no consumer exposure** **BYI02960-**	**exposure below TTC of 1.5 µg/kg bw** **BYI 02960-**
fatty acids	-acetyl-AMCP	-acetic acid
glucose	-acetyl-cysteinyl-nicotinic acid	-acetic acid-glyc
lactose	-AMCP-difluoroethanamine-SA	-amino-furanone
	-des-difluoroethyl	-bromo-amino-furanone
	-des-difluoroethyl-OH-SA	-bromo/-chloro
	-difluoroethyl-OH-glyc	-glyoxylic acid
	-hippuric acid	-AMCP-difluoroethanamine
	-lactato-mercaptyl-nicotinic acid	-N-acetyl-AMCP-difluoroethanamine
	-methylthio-glyoxylic acid	-N-formyl-AMCP-difluoroethanamine
		-mercapto-lactic acid

BYI 02960 metabolites		
covered by rat ADME **BYI 02960-**	**covered by aglycon** **BYI 02960-**	**with additional tox testings** **BYI 02960-**
-6-CNA	-6-CNA-glycerol-gluA	-CHMP
-OH	-OH-gluA	-difluoro acetic acid
	-OH-glyc	-difluoroethyl-amino-furanone
	-OH-SA	
	-cysteinyl-nicotinic acid	

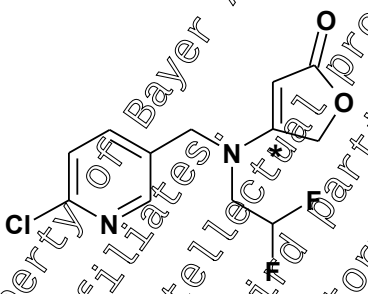
KIIA 5.11 – Summary of mammalian toxicity and overall evaluation

Absorption, distribution, excretion and metabolism

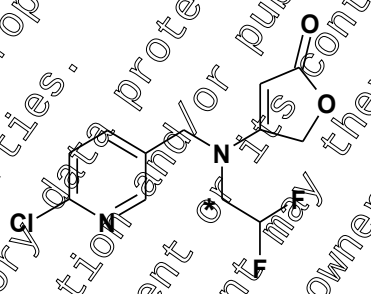
Absorption, distribution, excretion and metabolism of the new insecticide BYI 02960 (common name flupyradifurone) was investigated using three different labelling positions. The active substance was labelled with ^{14}C in the pyridinylmethylene bridge, in the 4-position of the furanone ring and in the 1-position of the ethyl side chain:



Pyridinylmethyl-^{14}C



Furanone-4-^{14}C



Ethyl-1-^{14}C

The pyridinylmethyl-labelled compound was used in an ADME-study in which male and female rats were orally administered with a low dose of 2 mg/kg and a high dose of 200 mg/kg. Due to the high water solubility of BYI 02960, male rats were also given an intravenous dose of 2 mg/kg. In this study, the excretion via urine and faeces was investigated as well as the distribution in the plasma and the radioactivity concentration in organs and tissues at sacrifice. The metabolism was investigated in urine and faeces.

A quantitative whole body autoradiography study was conducted also using the pyridinylmethyl-labelled compound following a single oral dose of 5 mg/kg to male and female rats. In this study the excretion of radioactivity was determined in urine, faeces and expired air as well as the distribution in the plasma and the radioactivity concentration in the organs and tissues at various time points.

The furanone-4-labelled compound was used in an ADME-study in which male and female rats were orally administered with 2 mg/kg. In this study, the excretion via urine and faeces was investigated as well as the distribution in the plasma and the radioactivity concentration in the organs and tissues at sacrifice. The metabolism was investigated in urine and faeces.

A quantitative whole body autoradiography study was also conducted using the furanone-4-labelled compound following a single oral dose of 5 mg/kg to male and female rats. In this study the excretion of radioactivity was determined in urine, faeces and the expired air as well as the distribution in the plasma and the radioactivity concentration in the organs and tissues at various time points.

In an organ metabolism study, male and female rats were orally administered with a single dose of 3 mg/kg [furanone-4-^{14}C]BYI 02960. Animals were sacrificed 6 h after dosage and the metabolism was investigated in urine, plasma, and in extracts of liver, kidney muscle and fat.

The ethyl-1-labelled compound was used in an ADME-study in which male rats were orally administered with 2 mg/kg. In this study, the excretion via urine, faeces and expired air was investigated as well as the distribution in the plasma and the radioactivity concentration in the organs and tissues at sacrifice. The metabolism was investigated in urine and faeces.

In an organ metabolism study, male and female rats were orally administered with a single dose of 3 mg/kg [ethyl-1-^{14}C]BYI 02960. The animals were sacrificed 1 h, 6 h, and 24 h after dosing. The total

radioactivity was determined at different time points in urine, while in plasma, liver, kidney, muscle and fat at sacrifice. The metabolism was investigated in urine, plasma, and in extracts of liver, kidney, muscle, and fat.

Following oral administration of a low dose of BYI 02960 to male and female rats, the gastrointestinal absorption of radioactivity was high. It accounted for >80 % of the dose independent of the labelling position used. Excretion was very fast, mainly renal and almost completed after 24 h. No radioactivity was detected in the expired air after dosing of the pyridinylmethyl- and ethyl-1-labelled compounds, proving the stability of these labelling positions in the molecule. Only after administration of [furanone-4-^{14}C]BYI 02960 between 1 and 3% of the administered radioactivity was exhaled. This demonstrated that for a small portion of the dose (higher in males than in females) the furanone ring of the molecule obviously was opened and underwent biotransformation to C-1 fragments.
The maximum plasma concentration was reached in most cases within 1 or 2 hours after administration of low doses. Only after administration of the high dose the peak plasma concentration was observed between 2 and 4 hours after dosage. After reaching the peak concentration, the radioactivity levels in plasma declined steadily by several orders of magnitude in all studies independent of sex or labelling position of the test compound.
Quantitative whole body autoradiography revealed a fast absorption and distribution of the test compound with peak values observed already 1 h after administration. At this time, the concentrations in liver and kidney were significantly higher than in blood, suggesting a preferred clearance from blood and distribution mainly to these organs which are mainly responsible for metabolism (liver) and excretion (kidney). Higher levels than in blood were also detected in the myocardium (heart) and in several glands. Very low levels were found in the brain, spinal cord and renal fat. These results are similar in male and female rats independent of the labelling position. A fast decline of radioactivity concentrations was observed for all organs and tissues in males and females during the entire test period. Concentrations fell for most organs and tissues below 5% of the maximum after one day. After seven days, only very low concentrations were found in a few organs and tissues of rats dosed with the pyridinylmethyl-labelled test compound. In the study using the furanone-4-labelled compound, low radioactive residues were measured in almost all organs and tissues due to the incorporation of C1- or C2-fragments into the endogenous carbon pool. The residues in males were higher by a factor of 1.4 to 4.7 as compared to females. A similar ratio of approx. 3 (males/females) was also found for the formation of $^{14}CO_2$. This is presumably due to sex related differences in metabolism leading to more C1- and C2-fragments and also higher incorporation of these components into the endogenous carbon pool in male rats. Basically males and female rats exhibited a very similar absorption, distribution and excretion behavior. The results of these studies demonstrate that there is no indication of any accumulation or significant retention of radioactivity in male and female rats. This observation is supported by the low P_{OW} of 1.2. Concentrations of radioactivity detected in tissues and organs at sacrifice were either very low or below the limit of detection.

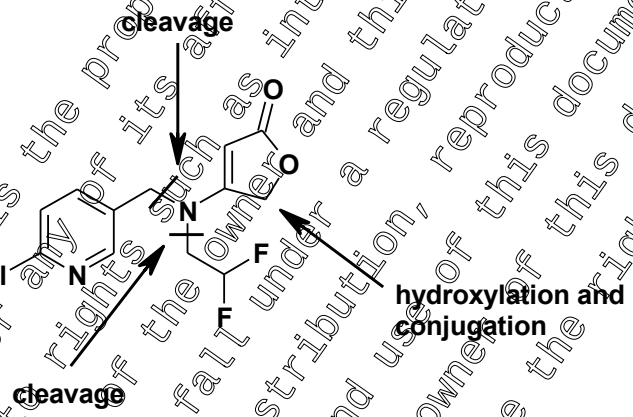
BYI 02960 was intensively metabolized in the rat. Numerous metabolites were formed, most of them being minor ones. The parent compound represented the predominant part of the radioactivity in urine of male and female rats. In faeces of male rats, the metabolite BYI 02960-OH was more prominent than the parent compound. Two metabolites, BYI 02960-6-CAN and BYI 02960-hippuric acid were also prominent in male but not in females rats.

The organ metabolism study using the ethyl-1-^{14}C label showed that in the 24 hours samples of plasma, and organs and tissues BYI 02960-DFA was by far the dominating metabolite accounting for more than 50% of the radioactivity.
The metabolic profiles in urine and faeces were very similar for both sexes but male rats showed a higher rate of metabolite formation as compared to female animals.

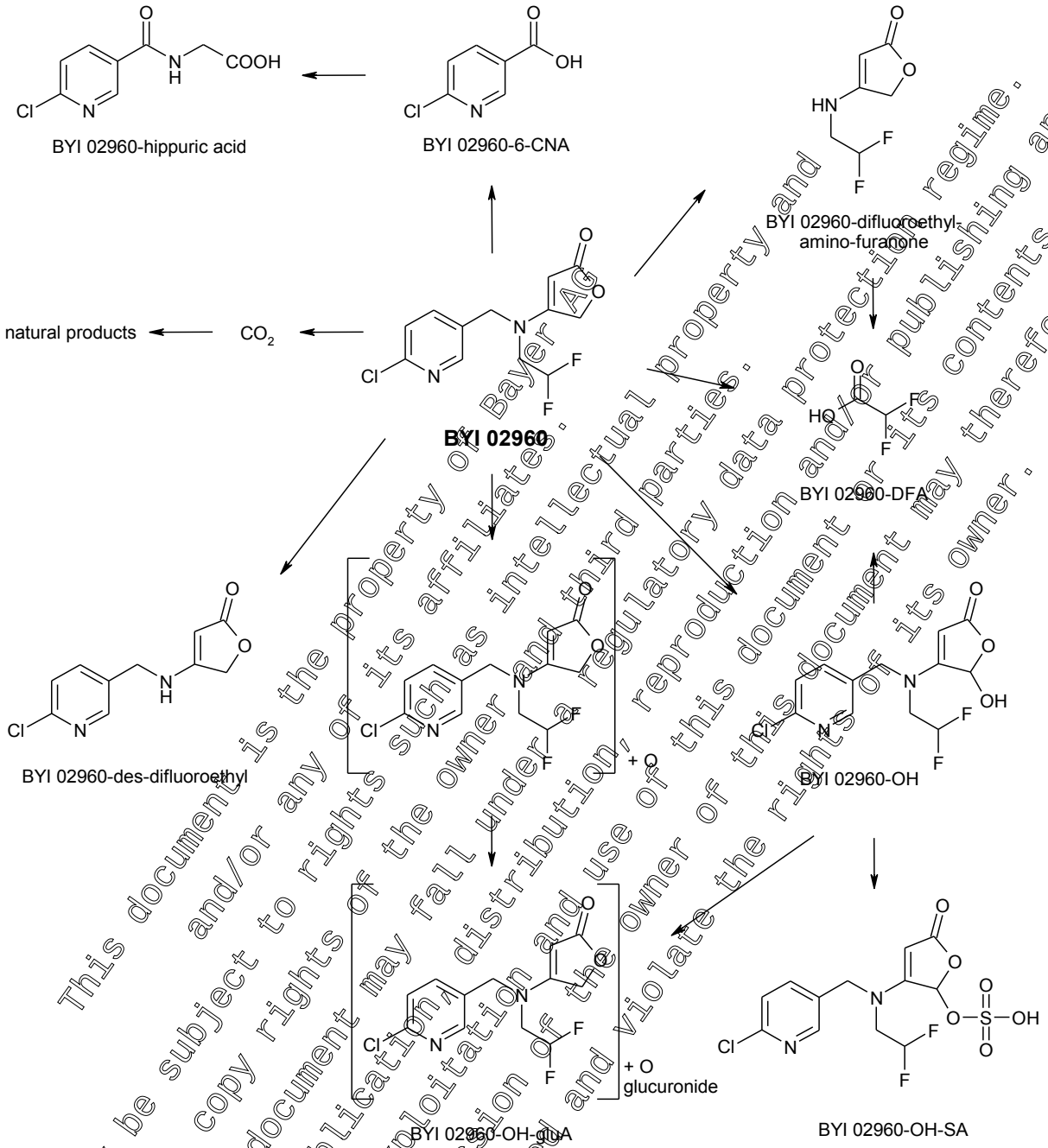
The principal metabolic reactions of flupyradifurone in rats were:

- Hydroxylation followed by conjugation with glucuronic acid or sulfate,
- cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl, and difluoroacetic acid (BYI 02960-DFA),
- cleavage of the molecule at the pyridinylmethylene bridge forming BYI 02960-6-CAN, which was further conjugated with glycine to BYI 02960-hippuric acid and BYI 02960-difluoroethyl-amino-furanone.

The figure below schematically shows the sites of the molecule, which are involved in the metabolic reactions:

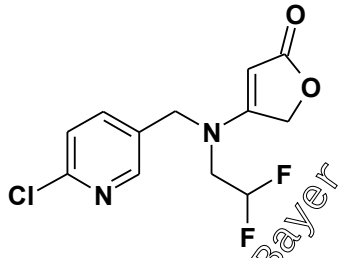
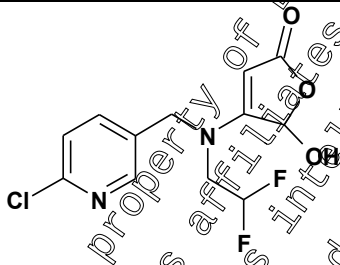
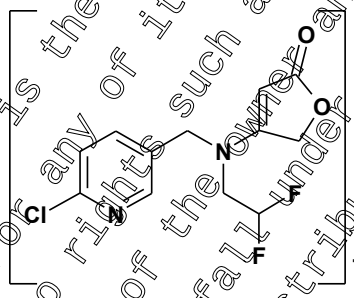
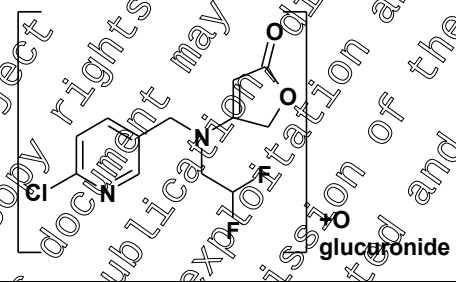
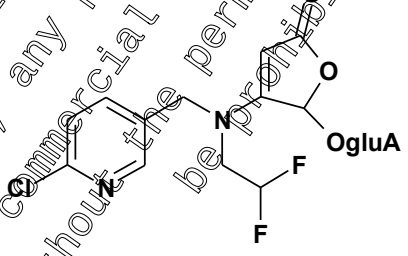


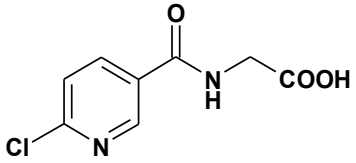
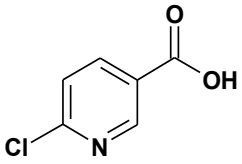
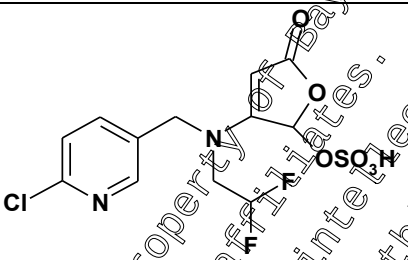
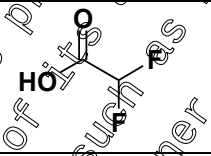
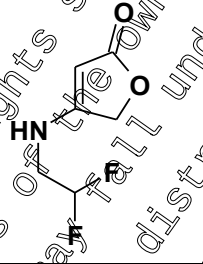
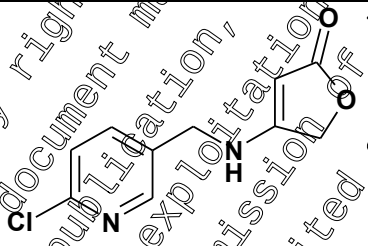
Summarizing the results of the metabolism studies conducted in the rat, a proposed metabolic pathway of BYI 02960 can be described as shown in this figure:



A comprehensive list of metabolites detected in the rat is provided in the following table.

Table 5.11-01: List of metabolites detected in the rat

Report Name	Chemical Structure	IUPAC Name
active substance: BYI 02960		4-[[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino]furan-2(5H)-one
BYI 02960-OH		4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-5-hydroxyfuran-2(5H)-one
BYI 02960-iso-OH		
BYI 02960-OH-gluA (isomer 1)		
BYI 02960-OH-gluA (isomer 3)		3-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-5-oxo-2,5-dihydrofuran-2-yl beta-D-glucopyranosiduronic acid

Report Name	Chemical Structure	IUPAC Name
BYI 02960-hippuric acid		N-[(6-chloropyridin-3-yl)carbonyl]glycine
BYI 02960-6-CNA		6-chloronicotinic acid
BYI 02960-OH-SA		3-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-5-oxo-2,5-dihydrofuran-2-yl hydrogen sulfate
BYI 02960-DFA		difluoroacetic acid [free acid]
BYI 02960-difluoroethyl-amino-furanone		4-[(2,2-difluoroethyl)amino]furan-2(5H)-one
BYI 02960-desdifluoroethyl		4-[(6-chloropyridin-3-ylmethyl)amino]furan-2(5H)-one

- **Acute toxicity, local tolerance and sensitization**

All studies were conducted in 2009, and were fully compliant with Good Laboratory Practice (GLP). All tests were conducted in accordance with prevailing OECD, EU, USEPA and Japanese MAFF testing guidelines.

The acute toxicity of BYI 02960 (96.2 % of purity) was low for all routes evaluated (oral, dermal and inhalational). The oral LD_{50} cut-off for rats was equal to 2000 mg/kg body weight (bw) with mortalities reported at 2000 mg/kg but none at 300 mg/kg. The rat acute dermal LD_{50} was > 2000 mg/kg bw. The

rat acute inhalation LC_{50} (4-hour) was > 4671 mg/m^3, which was the highest achievable concentration. There were no mortalities, but there were transient clinical signs which were reversible within 3 days.

BYI 02960 was not irritating to rabbit skin and caused only slight ocular irritation (redness of the conjunctivae) which reversed within 48 hours. No evidence of skin sensitization (delayed contact hypersensitivity) was seen in a modified LLNA test (IMDS) in NMRI mice.

Table 5.11-02: Summary of acute toxicity data for BYI 02960

Type of study (Document N°)	Species	Results	EPA Toxicity Category (proposed)	OECD Classification (proposed)
Oral route M-349992-01-2	Rat	Mortalities observed at 2000 mg/kg; none at 300 mg/kg	II	Category 4 (LD_{50} cut off = 2000 mg/kg)
Dermal route M-349995-01-2	Rat	LD_{50} > 2 000 mg/kg	III	Category 5 / Unclassified
Inhalation M-362791-01-2	Rat	LC_{50} at 4 hours > 4671 mg/m3	IV	Category 5 / Unclassified
Primary skin irritation M-353761-01-2	Rabbit	Non irritating	IV	Category 5 / Unclassified
Eye irritation M-361319-02-2	Rabbit	Slight redness of the conjunctivae, reversed within 48 hours	III	Category 5 / Unclassified
Skin sensitization M-353715-01-2	Mouse	Not sensitizing	Not Applicable	Category 5 / Unclassified

- **Short term toxicity**

The short-term toxicity studies with BYI 02960 were conducted between 2007 and 2011. Several range finding studies, which were not fully compliant to GLP, were performed in early phases. All compulsory studies were in accordance with OECD, EU, USEPA and Japanese MAFF testing guidelines and were fully compliant with GLP. A summary of all these results is presented in Table 5.3-01.

Two 28-day rat studies were performed, one using a gavage administration and the other an administration through the diet. In the first gavage study, wistar rats (5/sex/group) were administered at 75, 200 and 350 mg/kg/day. The vehicle used was corn oil supplemented with 10% ethanol and 10% water, v/v. Two females died on day 6 at 350 mg/kg/day. Not statistically significant lower mean body weight was observed at 350 mg/kg/day in the males throughout the study and in females during the first week. Lower total bilirubin and glucose concentrations were observed in both sexes at 350 mg/kg/day and in males only at 200 mg/kg/day. Higher triglyceride concentration was observed in both sexes at 350 mg/kg/day and in females only at 200 mg/kg/day. An increase in creatinine concentration and alanine aminotransferase and/or alkaline phosphatise activities were noted in females at 350 and 200 mg/kg/day. The target organs were the liver and the thyroid with higher absolute and/or relative liver weights and centrilobular hepatocellular hypertrophy observed in both sexes at 350 and 200 mg/kg/day and diffuse follicular cell hypertrophy observed in the thyroid in both sexes at 350 mg/kg/day and in males only at 200 mg/kg/day. BYI 02960 showed a cytochrome P450 3A family inducer profile with an increase in

BROD activity observed in both sexes at 350 and 200 mg/kg/day. The NOAEL of this study was 75 mg/kg/day for both sexes.

As there was no significant differences between males and females in the first study, only male Wistar rats (5/group) were used in the second study. BYI 02960 was administered through the diet at 500 (actual analyzed concentration of 410 ppm equivalent to 33.6 mg/kg body weight/day) and 5000 ppm corresponding to 385 mg/kg body weight/day. Treatment-related findings were only observed at 5000 ppm. Lower mean body weight and food consumption were observed throughout the study period. Lower total bilirubin and glucose concentrations and higher urea and total cholesterol concentrations were observed at the end of the study. Hormone analysis showed an increase in TSH and a slight decrease in T4. The target organs were both the liver (with increased relative weight, prominent lobulation and centrilobular hepatocellular hypertrophy) and the thyroid gland (with diffuse follicular cell hypertrophy). Both BROD and UDPGT activities were increased at the end of the study. The NOEL in this study was 410 ppm equating to 33.6 mg/kg/day.

Wistar rats (10/sex/group) were administered at 100, 500 and 2500 ppm (equating to 6.0, 30.2 and 156 mg/kg/day in males and 7.6, 38.3 and 186 mg/kg/day in females) for at least 90 days. An additional 10 animals per sex were fed control or high dose test diet for at least 90 days and subsequently fed control diet and observed for reversibility or persistence of toxic effects after a post-treatment recovery period of at least 28 days. Significant findings were limited to the group treated at 2500 ppm, except for a reduced mean body weight gain observed in females during the first and the last weeks of the study. At 2500 ppm, a lower body weight was observed in both sexes throughout the study. Throughout the recovery phase of the study the mean body weight of males and females remained lower than the control group. A slight reduction in mean food consumption was observed for males during the first four days of the study and thereafter on several occasions and for the females from the first week of the study until Study Week 7. A higher mean platelet count was observed in females. Mean total bilirubin and glucose concentrations were slightly lower in both sexes and mean total cholesterol and triglycerides concentrations were slightly higher when compared to the controls. The change observed for total bilirubin was considered to be partially reversible in females. The other treatment-related changes were considered to be reversible. The target organs were also the liver (with higher relative weights to body weight ratio and centrilobular hepatocellular hypertrophy in both sexes) and the thyroid gland (dark aspect at necropsy and minimal follicular cell hypertrophy in some males). These findings were totally reversible. The NOAEL in this study was 500 ppm equating to 30.2 and 38.3 mg/kg/day in males and females, respectively.

In a 28-day mouse study, BYI 02960 was administered to C57BL/6J mice at 300, 600 and 1200 ppm (equating to 50, 98 and 207 mg/kg/day in males and 59, 122 and 240 mg/kg/day in females). The only effect observed in this study was a slightly lower mean body weight in males on study day 8 at 1200 ppm. The NOAEL was considered to be 1200 ppm. Due to instability of BYI 02960 in rodent diet the actual concentration is considered to be in the region of 960 to 1080 ppm (166 to 186 mg/kg body weight/day for the males and 192 to 216 mg/kg body weight/day for the females, respectively).
In the 90-day mouse study, BYI 02960 was administered to C57BL/6J mice at 100, 500 and 2500 ppm (equating to 16, 81 and 407 mg/kg/day in males and 19, 98 and 473 mg/kg/day in females). Effects were limited to 2500 ppm except that lower mean body weight gain was observed in males during the first week of the study. A lower body weight was observed in both sexes throughout the study. A slight

reduction in mean food consumption was observed in females between Study Days 1 and 22. A lower mean total cholesterol concentration, higher mean urea concentrations and a slightly lower total protein concentrations were observed in both sexes, whilst higher mean alkaline phosphatase activity was noted in males and mean alanine and aspartate aminotransferase activities were higher in females. In females, mean albumin concentrations were slightly lower. The target organs were the liver (with higher mean absolute and relative weights in females, pale liver in the females and slight increase in severity of diffuse hepatocellular vacuolation in both sexes) and the kidney (with lower mean absolute and relative weights to brain weight ratio in males and loss of the normal multifocal/diffuse cortical epithelial vacuolation in males also). The NOAEL was 500 ppm (equating to 80.6 mg/kg body weight/day) in males and the NOEL in females (equating to 98.1 mg/kg body weight/day).

Groups of two males and two females Beagle dogs received BYI 02960 mixed in their diet at concentrations of 0, 500, 2000 or 4000 ppm (equating approximately to 0, 16, 62, 118 mg/kg body weight/day in males and 0, 18, 77, 131 mg/kg body weight/day in females) for at least 28 days. At 4000 ppm, there was an overall body weight loss observed in one male whilst the body weight of the other male remained static. One female had an overall body weight gain of 0.7 kg whilst the body weight of the other female remained static. Lower food consumption was observed in both male and female animals. Hematology assessment revealed a slightly increased platelet count in both females and in 1/2 males at 4000 ppm and in 1/2 females at 2000 ppm. In isolation this treatment-related change was not considered to be adverse. The target organ was the liver with decreased centrilobular glycogen accumulation in incidence and/or severity in both sexes at 4000 ppm and in males only at 2000 ppm. This was considered to be a treatment-related but not adverse effect. The NOAEL in this study was 2000 ppm (equating to 62 and 77 mg/kg/day in males and females, respectively).

BYI 02960 was administered via the diet to beagle dogs (4/sex/dose) of both sexes at nominal concentrations of 0, 400, 1200 or 3600/2400 ppm for at least 90 days (equating approximately to 12, 33 or 102/85 mg/kg body weight/day in males and 12, 41 or 107/78 mg/kg body weight/day in females). The 3600 ppm dose group was reduced to 2400 ppm beginning Study Week 9 due to clinical signs seen in two of the dogs on Day 44 and continual weight loss in the high-dose group.
In the high dose group, compound-related clinical findings were unsteady and stiff back legs and lower back on study days 44, 53, and 54 in one male and on study day 44 for one female. Lower body weight was observed in males and females during the first week of the study at 3600/2400 ppm and in males only at 1200 ppm. Food consumption was also reduced at the beginning of the study in both sexes at 3600/2400 ppm and in males only at 1200 ppm. Higher creatine phosphokinase, aspartate aminotransferase, and alanine aminotransferase activities were observed at the 2-month test interval in both sexes at 3600/2400 and 1200 ppm. Lower red blood cell count, hemoglobin concentration, and hematocrit were observed at 3600/2400 ppm at 1, 2, and 3 months in both sexes. The target organs were the liver at 3600./2400 ppm (with higher absolute and relative weights in both males and females and minimal brown pigments in Kupffer cells in females), the kidney (with higher relative weights in both sexes at 3600/2400 ppm and males only at 1200 ppm) and skeletal muscle (with minimal to slight myofiber atrophy/degeneration in both sexes at 3600/2400 ppm and 1200 ppm). The NOEL in this study was 400 ppm for males and females equating to 12 mg/kg/day.

Male and female Beagle dogs (4/sex/dietary level) were fed control feed or feed containing BYI 02960 at dietary concentrations of 150, 300, or 1000 ppm (approximately equal to 4.6/4.1, 7.8/7.8, 28.1/28.2

mg/kg body weight/day in males/females, respectively) for at least one year. Test substance-related effects were limited to degeneration noted in skeletal muscle (gastrocnemius, biceps femoris) of males and females at 1000 ppm only. Minimal to slight, focal to multifocal areas of degeneration of skeletal muscles were noted in males (gastrocnemius - incidence 2/4; biceps femoris - incidence 3/4) and females (gastrocnemius - incidence 2/4; biceps femoris - incidence 3/4). Degeneration of the myofiber comprised one or more of the following changes: atrophy, necrosis, and/or presence of inflammatory cells around the affected myofiber. Skeletal muscles evaluated from 150 ppm and 300 ppm males and females were comparable to controls. Based on the micropathology findings, the lowest-observed-adverse-effect-level (LOAEL) in this study was 1000 ppm, which was equivalent to 28.5 and 29.3 mg/kg body weight/day for male and female dogs, respectively. Based on the lack of adverse compound-related effects, a dietary level of 300 ppm (equivalent to 7.8 mg/kg body weight/day for both sexes) was considered to be a no-observed-adverse-effect-level (NOAEL).

Male and female Wistar rats (10/sex/group) were administered vehicle or BYI 02960 (50, 150 or 500 mg/kg/day) daily by dermal application for at least 28 consecutive days (minimum of 6 h/day) and euthanized one day following the last dose. The toxicological response of the rat was principally characterized by non-adverse decreases in food consumption in female rats at 500 mg/kg/day in the first two weeks and at 50 mg/kg/day in the second week and mild decreases in absolute and relative liver weights in males with no clinical pathology or micropathology correlates. The No-adverse-effect-level in this study was 500 mg/kg/day for both male and female rats.

Table 5.11-03: Summary of short-term toxicity of BYI 02960

Type of study (Document N°) Concentrations in feed	NOEL/NOAEL		LOAEL		Adverse effects at high dose levels
	ppm	mg/kg/d	ppm	mg/kg/d	
28-day rat study (M-283421-02-2) 0, 75, 200 & 350 mg/kg/day	-	75	-	200	Liver: centrilobular hepatocellular hypertrophy, both sexes Thyroid: Minimal diffuse follicular cell hypertrophy in males only at 200 mg/kg/day
28-day rat study (M-297120-01-2) 0, 500 & 5000 ppm	500	33.6	5000	385	Liver: slight to moderate diffuse centrilobular hepatocellular hypertrophy Thyroid: Minimal to slight diffuse follicular cell hypertrophy Decreased T4, increased TSH, BROD and UDPGT inductions
90-day rat study (M-329048-03-2) 0, 100, 500 & 2500 ppm	500	30/38	2500	156/186	Liver: centrilobular hepatocellular hypertrophy in both sexes Thyroid: follicular cell hypertrophy in males only
28-day mouse study (M-294820-01-2) 0, 300, 600 & 1200 ppm	960 to 1080	166 to 186	>960 to 1080	>166 to 186	Only slight body weight decrease
90-day mouse study (M-328668-03-2) 0, 100, 500 & 2500 ppm	500	80.6/98.1	2500	407/473	Liver: increased diffuse hepatocellular vacuolations Kidney: decreased multifocal/diffuse Corticoepithelial vacuolation :
28-day dog study (M-312461-01-3) 0, 500, 2000 & 4000 ppm	2000	62/77	4000	118/131	Liver: centrilobular glycogen accumulation decreased in incidence and/or severity
90-day dog study (M-369978-01-2) 0, 400, 1200 & 3600/2400 ppm	400	12/12	1200	33/41	Liver: increased absolute and relative weight in both sexes; brown pigment in Kupffer cells in females (high dose) Kidney: increased relative weights in both sexes Skeletal muscle: myofiber atrophy/degeneration in both sexes
1 year dog study (M-425272-01-2) 0, 150, 300, 1000 ppm	300	7.8/7.8	1000	28.1/28.2	Minimal to slight degeneration of skeletal muscle (gastrocnemius and biceps femoris) in both sexes
28-day dermal rat study (M-432336-01-1) 50, 150, 500 mg/kg/d	500		> 500		Non-adverse decreases in food consumption in females and mild decreases in absolute and relative liver weights in males

- **Genotoxicity**

BYI 02960 was tested in a standard battery of *in vitro* and *in vivo* genotoxicity studies and mutagenicity tests *in vitro* and *in vivo* carried out according to the current OECD and European guidelines. The studies were performed between 2009 and 2011 in compliance with GLP requirements. There was no indication of gene mutation in either the presence or absence of metabolic activation in both the bacterial reverse mutation and mammalian gene mutation tests. The *in vitro* chromosome aberration test

and the *in vivo* mouse micronucleus tests were also both negative. These studies demonstrate that BYI 02960 has no genotoxic potential.

Table 5.11-4: Summary of genotoxicity test

Mutagenicity tests with BYI 02960	Metabolic Activation	Results
A. *In vitro* tests		
Ames Test (M-354173-01-2)	+/-	Negative
Ames Test (M-420539-02-2)	+/-	Negative
Chromosome aberrations (V79 cells) (M-359746-01-2)	+/-	Negative
HPRT Test (V79 cells) (M-359743-01-2)	+/-	Negative
B. *In vivo* tests	**Dose levels**	
Micronucleus Test in male mice - oral administration (M-353785-01-2)	10, 20 and 40 mg/kg	Negative
Micronucleus Test in female mice - oral administration (M-420536-01-2)	12.5, 25 and 50 mg/kg	Negative

- **Long term toxicity and carcinogenicity**

The oncogenic potential of BYI 02960 was assessed in both the rat and the mouse. The studies were conducted between 2009 and 2012. All were in accordance with OECD, EU, USEPA and Japanese MAFF testing guidelines and were fully compliant with GLP. A summary of these results is presented in Table 5.5-01.

In the rat combined chronic toxicity and carcinogenicity study, where the animals were administered BYI 02960 through the diet at up to 2000 ppm, lower body weight and body weight gain were observed in females at 2000 ppm throughout the study and slightly lower cumulative body weight gain was observed in males during the first year. Higher mean leukocyte counts associated with higher mean absolute lymphocyte and neutrophil counts were observed in males from the end of the first year. Slightly higher cholesterol concentrations were seen in the females throughout the study. No relevant treatment-related neoplastic changes were observed at any dose level tested. The target organs were the liver and the thyroid in either sex and the lung in females. The effects seen in the liver in the 2000 ppm treated male and female groups were higher mean liver to body weight ratios associated with centrilobular hypertrophy, centrilobular hepatocellular macrovacuolation, lower incidences of periportal hepatocellular vacuolation and eosinophilic, mixed and tigroid foci of altered hepatocytes. In addition, higher incidences of brown pigments in Kupffer cells, interstitial mononuclear cell infiltrate and periportal hepatocellular macrovacuolation were observed in females. Changes were also observed in the thyroid gland including higher incidences of follicular cell hypertrophy and of follicular cell pigment in both sexes at the final sacrifice and increased incidences of colloid alteration in males and females at the interim sacrifice and in males only at final sacrifice. In the lung, higher incidences of foamy macrophages and chronic interstitial and perivascular inflammation were observed in females at final sacrifice. At 400 ppm, the findings were limited to centrilobular hypertrophy (minimal) in the liver

and of colloid alteration in the thyroid gland observed in males. However these changes were considered not to be adverse since they were minimal and/or not associated with other relevant changes.

The No Observed Adverse Effect Level over a 12-month period of dietary administration with BYI 02960 to the Wistar rat was 400 ppm in both sexes (equivalent to 18.5 mg/kg body weight/day in males and 25.3 mg/kg body weight/day in females).
The No Observed Adverse Effect Level over a 24-month period of dietary administration with BYI 02960 to the Wistar rat was 400 ppm in both sexes (equivalent to 15.8mg/kg/day weight/day in males and 22.5 mg/kg body weight/day in females).

In the mouse carcinogenicity study, where animals were administered BYI 02960 through the diet up to 1500 ppm, mean body weight was progressively decreased in both sexes throughout the study compared to control means. No relevant treatment-related neoplastic changes were observed at any dose level tested. The target organs were the liver and the kidney. The changes observed in the liver were higher liver weights and a higher incidence and severity of diffuse hepatocellular vacuolation (mainly centrilobular) noted in males, whilst a decreased incidence of diffuse hepatocellular macrovacuolation (mainly periportal) was noted in females. In the kidney, lower weight, decreased incidence and severity of bilateral basophilic tubules, focal cortical mineralization and corticoepithelial vacuolation were noted in males. These changes in the kidney of the liver were considered to be treatment-related but not adverse. At 300 ppm, the only histopathological changes were noted in males and consisted of a higher incidence and severity of diffuse hepatocellular vacuolation (mainly centrilobular) in the liver together with a decreased severity of corticoepithelial vacuolation in the kidney. Both changes were considered to be treatment-related but not adverse.

A dose level of 300 ppm (equivalent to 43 mg/kg/day in males and 53 mg/kg/day in females) was considered to be a No Observed Adverse Effect Level in both sexes over an 18-month period of dietary administration.

Table 5.11-05: Summary of long-term toxicity/carcinogenicity with BYI 02960

Type of study Doses	NOAEL mg/kg/day	LOAEL mg/kg/day	Effects
Rat - 104-week Chronic Toxicity/ Oncogenicity M-428257-01-1 80, 400 & 2000 ppm	15.8/22.5 (M/F)	80.8/120 (M/F)	Target organs: liver & thyroid either sex; lung in females No tumours
Mouse - 78 week- Chronic/ Oncogenicity M-425975-01-1 70, 300 & 1500 ppm	43/53 (M/F)	224/263 (M/F)	Target organs: liver either sex; kidney in males No tumours

- **Mechanism of action and supporting data**

As no treatment-related tumours were observed in both rats or mice, no mechanistic studies were undertaken.

- **Reproductive and embryonic toxicity**

All studies presented in this section were conducted between 2010-2012 and complied with OECD, EU, USEPA and Japanese MAFF testing guidelines and Good Laboratory Practice (GLP).

In the rangefinder one generation rat reproduction study, BYI 02960 was administered continuously in the feed to Wistar rats (10 animals/dose/sex) at nominal dietary concentrations of 0, 200, 700, and 2000 ppm.: Males exhibited a very slight decline in body weight gain over 15 weeks of treatment with the test substance at 2000 ppm. Females showed declines in absolute body weight and body weight gain as well as declines in food consumption throughout the premating period at 2000 ppm and decline in body weight gain at 700 ppm. Statistically significant body weight declines were also observed throughout gestation and lactation at 2000 ppm; at 700 ppm declines in body weight during lactation with significance observed by lactation Day 14 were observed. Females treated at 2000 ppm also exhibited test substance-related decreases in absolute and relative spleen weight. At 2000 and 700 ppm, declines in absolute male and female pup weight were observed beginning PND 14 and continuing to PND 21 with significance only observed for the females. Body weight gain for the males and females was also declined, relative to controls. No test substance-related findings were observed on reproductive parameters.

In the rat two-generation reproduction study, BYI 02960 was administered continuously in the diet to Wistar rats (30 animals/dose/sex) at nominal dietary concentrations of 0, 100, 500, and 1800 ppm. In the P-generation and F_1-generation, females from the 1800 ppm treated group exhibited declines in body weight during premating, gestation and laction. In the P-generation males treated at 1800 ppm, increased absolute and relative liver weights were observed as well as increased absolute thyroid weights. Minimal centrilobular hypertrophy of the liver was observed in the males and correlated with the increased liver weights. Declines in body weight were also observed in the females treated at 500 ppm from the P-generation during the premating period and the females from the F_1-generation during premating, gestation and lactation periods.
F_1-offspring from the 1800 ppm parental group showed a significant decline in body weight at birth and during lactation. No decline in body weight was observed at birth for the F_2-offspring, but a significant decline was observed during lactation. In the F_1-offspring a significant delay in preputial separation and a slight nonstatistical delay in vaginal patency were observed in parallel with the decreased body weight. However, no effect on anogenital distance was observed in the F_2-generation pups. These findings are considered to be a consequence of the decreased pup weight exhibited during lactation in this same dose group rather than an endocrine-mediated mode of action. This explanation is supported by the similar mean body weight values on the day of complete PPS between controls (178 g) and the 1800 ppm group (180g). Similarly, the apparent delay in vaginal patency (by 1 day) in the 1800 ppm group is strongly correlated to decreased body weight and body weight gain during lactation as well as decreased maternal body weight parameters throughout the study. Additional support is seen in the lack of treatment-related effects on other endocrine-sensitive parameters in both males and females. In both generations, variations in brain, thymus and spleen weights in males and/or females were observed and are considered to be due to the decreased body weights observed at this same dietary level and not a direct effect of the test substance. At 500 ppm a decline in body weight was observed in F_2-generation pups. Variations in brain, thymus and spleen weights in males and/or females were also observed in the F_2-offspring and are considered to be due to the decreased body weights.

A slight decrease in litter size was noted in the F_2-generation pups at 1800 ppm. The decline in litter size (9.2) is just outside of this laboratory's historical control range (9.8 - 11.8) and declines in total gain during gestation for the F_1-adults occurred concomitantly with this finding. Also, associated with the decrease in litter size was a decline in the total number of implantation sites in the F_1-adults, relative to controls. There was no test substance-related effects observed on the viability of the pups after delivery at any dietary level tested.
The parental systemic NOAEL was 500/100 ppm in males and females, respectively (32.3/7.8 mg BYI 02960/kg bw/day) based upon liver and thyroid effects in P-generation males and body weight effects in females. The reproductive NOAEL was 500 ppm (32.3/39.2 mg BYI 02960/kg bw/day in males and females, respectively) based upon decreased cycle number, litter size and number of implants in F_1 generation. The offspring NOAEL was 100 ppm (7.8 mg BYI 02960/kg bw/day) based upon body weight effects in F_2 pups.

In a rat developmental study, BYI 02960 was administered daily by gavage to groups of 25 pregnant Sprague-Dawley female rats per dose-group at 15, 50 and 150 mg/kg/day from gestation day (GD) 6 to 20. The control group received the vehicle alone, an aqueous solution of 0.5% methylcellulose 400. At 150 mg/kg/day, there was a mean maternal body weight loss of 5.7 g between GD 6-8 compared to a weight gain of 5.9 g in the concurrent controls. In addition, between GD 8-10, the mean body weight gain was reduced by 24% when compared to the control group. Mean food consumption was reduced by between 9 and 27% on all intervals between GD 6 and 12. At 50 mg/kg/day, the mean maternal body weight gain was reduced by 49% and mean food consumption was reduced by 8% between GD 6 - 8, when compared to the concurrent controls. At 150 mg/kg/day, the mean absolute liver weight was 13% higher than controls. At cesarean section, mean fetal body weights for combined sexes and females were marginally reduced compared to the controls (by 2 to 3%, not statistically significant). At the fetal skeletal examination, the incidences of two variations ("parietal (uni/bi): incomplete ossification" and "hyoid centrum: incomplete ossification") were higher than in the control group and were indicative of a slightly delayed fetal development. The NOEL for maternal toxicity was 15 mg/kg/day and the NOEL for developmental toxicity was 50 mg/kg/day.

In a complementary study, where groups of 23 sperm-positive female Sprague-Dawley rats were exposed to BYI 02960 by oral gavage from gestation day (GD) 6 to 20 at 20 and 30 mg/kg/day, no maternal toxicity was observed up to 30 mg/kg/day. Therefore, based on these two studies, it can be concluded that the NOAEL for maternal toxicity was 30 mg/kg/day and the NOAEL for developmental toxicity was 50 mg/kg/day.

In a rabbit developmental study, groups of 23 time-mated pregnant female New Zealand White rabbits were administered BYI 02960 by oral gavage from gestation day (GD) 6 to 28 at 7.5, 15 and 40 mg/kg/day. A dose level of 40 mg/kg/day BYI 02960 resulted in maternal toxicity as evidenced by body weight loss, significantly reduced body weight gain and food consumption between GD 6 and 10, and lower mean maternal corrected body weight change compared to control animals. Fetal development was unaffected by treatment at any dose level tested. A dose level of 15 mg/kg/day was considered to be a NOAEL for maternal toxicity, while a dose level of 40 mg/kg/day was considered to be a NOAEL for developmental toxicity.

Table 5.11-06: Summary of the reproductive and embryonic toxicity studies

Type of study Doses	NOAEL (mg/kg/d)	LOAEL (mg/kg/d)	Adverse effects at LOAEL/ target organs	
Reproductive toxicity studies				
One-generation rat M-394208-01-2 0, 200, 700, 2000 ppm	**50.1/17.5** (M/F)	**147.5/60** (M/F)	Parent	*Males:* Slight declines in BWG *Females:* Decreased BW and /or BWG (premating, gestation, and lactation)
	147.5/168.9 (M/F)	**>147.5/168.9** (M/F)	Repro-duction	No effects
	17.5	**60. 9**	Offspring	Decreased BW and BWG
Two-generation rat M-417665-01-2 0, 100, 500, 1800 ppm	**32.3/7.8** (M/F) [500/100 ppm]	**119.8/39.2** (M/F) [1800/500 ppm]	Parents	*Males:* Increased liver weights (P) Increased thyroid weights (P) Increased incidence of centrilobular hypertrophy (minimal - P) *Females:* Decreased BW (premating, gestation, and lactation; F_1) Decreased BWG (premating; P and F_1) Decreased terminal body weights (P & F_1)
	32.3/39.2 (M/F) [500/500 ppm]	**119.8/140.2** (M/F) [1800/1800 ppm]	Repro-duction	Decreased cycle number (F_1), litter size (F_1), and number of implants (F_1)
	7.8 (M/F) [100 ppm]	**39.8** (M/F) [500 ppm]	Offspring	Decreased BW and BWG (F_2); with Secondary to BW decreases: organ weight changes in brain, thymus, and spleen
Developmental toxicity studies				
Developmental toxicity Rat M-363938-01-2 0, 15, 50, 150 mg/kg/d	**15** (Maternal)	**50**	Dams	Decreased mean BWG and food consumption (FC)
	50 (Develop.)	**150**	Fetuses	Decreased fetal BW; Reduced ossification of a few skull bones
Complementary rat toxicity M-425810-01-2 0, 20, 30 mg/kg/d	**30**	**>30**	Dams	No maternal toxicity
Developmental toxicity rabbit, M-423559-01-1 0, 7.5, 15, 40 mg/kg/d	**15** (Maternal)	**40**	Dams	Decreased BW, BWG, corrected BWG, and FC (GD6-10)
	40 (Develop.)	**>40**	Fetuses	No treatment-related effects

- **Neurotoxicity**

All studies presented in this section were conducted between 2009 and 2011 and complied with the EU, OECD USEPA and Japanese MAFF testing guidelines and Good Laboratory Practice (GLP).

In an acute neurotoxicity study, technical grade BYI 02960 was administered by gavage in a single dose to non-fasted young adult Wistar rats at 0, 50, 200 and 800 mg/kg. Compound-related effects were observed at all dose concentrations in both sexes. Findings associated with treatment at the time-of-peak effect after dosing included piloerection, lower muscle tone, rapid respiration, low arousal, tremors, myoclonic jerks, chewing, repetitive licking of lips, gait incoordination, flattened or hunched posture, dilated pupils, impaired (uncoordinated or slow) righting reflex, impaired flexor and tail pinch responses and reduced rectal temperature. Automated measures of motor activity were also reduced in both sexes, compared to controls. The only treatment-related effects at 50 mg/kg were limited to higher incidences of piloerection in both sexes and dilated pupils in females only. A follow-up study was performed in order to establish a clear NOAEL for findings observed at all dose levels in the initial study. In this follow-up study, females only were used as they were equal or more sensitive than males at higher dose levels and were administered BYI 02960 at 20 or 35 mg/kg. No treatment-related effects were evident at either dose tested. The dose level of 35 mg/kg of BYI 02960 was considered to be the overall NOAEL for both sexes.

In a 90-day neurotoxicity study, through approximately 13 weeks of continuous dietary exposure to BYI 02960 at 100, 500 or 2500 ppm, there were no neurotoxic treatment-related findings apparent at any dietary level in either sex. Based on these findings, a NOAEL of 2500 ppm was established for the rat (equating to 143 and 173 mg BYI 02960/kg body wt/day for males and females, respectively).

In the developmental neurotoxicity study, BYI 02960 was administered via the diet from gestation Day (GD) 6 trhough lactation Day (LD) 21 to mated female Wistar rats at nominal concentrations of 0, 120, 500 or 1200 ppm with adjustment during lactation to maintain a more consistent dosage throughout the period of exposure. In maternal animals, body weight was statistically reduced at 1200 ppm on GD 20, LD 0 and LD 4, with non-statistical decreases that continued through termination on LD 21. In offspring at the same concentration, body weight was non-statistically reduced on PND 17 and 21 in both sexes. Body weight gain was statistically decreased in males and in both sexes combined for PND 4-17 and in males and females for PND 11-17. Startle amplitude was statistically increased in females on PND 60. Average session motor and locomotor activity was non-statistically increased in males on PND 13. There were no treatment-related effects in maternal animals or offspring at 500 and 120 ppm. Therefore, the NOAEL for both maternal animals and offspring is 500 ppm (42.4 mg/kg/day) in this study.

Table 5.11-07: Summary of neurotoxicity with BYI 02960

Type of study (Document N°) Doses	NO(A)EL (mg/kg/d)	LOAEL (mg/kg/d)	Adverse effects / target organs
Acute neurotoxicity in the rat M-415408-01-2 0, 20, 35, 50, 200 and 800 mg/kg bw	35 (M/F)	50 (M/F)	Piloerection and dilated pupils - At high dose levels: lower muscle tone, rapid respiration, gait incoordination, tremors, reduced motor activity, impaired righting reflex, impaired flexor and tail pinch responses
90-day neurotoxicity in the rat M-410022-01-2 0, 100, 500,2500 ppm	143/173 (M/F)	> 143/173 (M/F)	None
Developmental neurotoxicity in the rat M-434203-01-1 120, 500, 1200 ppm	424	102	↓ Body weight and body weight gain in dams ↓ Body weight and body weight gain in pups during lactation ↑ Startle amplitude (females only) on PND 60 ↑ Motor and locomotor activity on PND 13 (males only)

- **Toxicity of metabolites**

Toxicology study programs for plant and environmental metabolites of BYI 02960 have been performed in accord with EU guidance, with all studies carried out according to current OECD, EU, USEPA and Japanese MAFF testing guidelines. The toxicological properties of two metabolites specific to BYI 02960 and two additional metabolites which are also formed from other agrochemicals are reported in this section.

Difluoroacetic acid (DFA, BCS-AA56716) is a major soil, water and plant metabolite of BYI 02960; in the rat ADME study, it was found in urine at around 6% of the administered dose. This metabolite is devoid of genotoxic potential; the acute oral LD50 is between 300 and 2000 mg/kg, similar to parent compound. In a 14 day repeat dietary administration range finding study in the rat, the most significant findings were decreased mean glucose concentration in both sexes and an increase in urea concentration was observed in females only. In a 90-day rat study, DFA was administered in the diet to Wistar rats (10/sex/group) at concentrations of 200, 1000 and 6000 ppm. Lower mean glucose concentrations, lower total bilirubin and slightly higher mean urea concentrations were observed in both sexes at all doses. At 6000 and 1000 ppm dose levels, mean body weight, overall body weight gain and food consumption were reduced in both sexes. Lower hemoglobin concentration and lower mean corpuscular volume were observed in females, together with lower mean corpuscular hemoglobin and lower hematocrit, and higher ketone levels were noted in both sexes. A few black foci were also noted in the glandular part of the stomach in both sexes (including one control female), in correlation with a few cases of focal glandular erosion/necrosis observed at the microscopic examination. The minor changes noted in the clinical chemistry determination at the low dose are considered not to be adverse effects of

the test substance as they do not represent any functional impairment in the test organism. Therefore, the dose level of 200 ppm (equating to 12.7 and 15.6 mg/kg body weight/day in males and females, respectively) was considered to be a No Observed Adverse Effect Level (NOAEL) in the male and female Wistar rats. When the NOAEL is expressed in BYI 02960 equivalents, it equates to 38 and 47 mg/kg/day in males and females, respectively. Therefore, Difluoroacetic acid was not more toxic than BYI 02960 after subchronic administration to the rat. The metabolic changes observed with DFA are also observed with BYI 02960. The decrease in glucose was reversible and appeared to be adaptative as it was no longer significant during the second part of the rat carcinogenicity study.

BYI 02960-difluoroethyl-amino-furanone (BCS-CC98193, BYI 02960-DFEAF) is a minor plant metabolite of BYI 02960 and was also observed in the rat ADME study. It accounted for less than 10% of the administered dose in the rat urine. Since the confined rotational crop study with [furanone-4-14C]BYI 02960 indicated rather high residue levels of BYI 02960-difluoroethyl-amino-furanone in leafy crops and the absence of other suitable markers, BCS decided to include this metabolite in the residue definition for the data collection method for target and rotational crops (see AII 6.7.1 and AII 6.11.1). The subsequent residue studies revealed that BYI 02960-difluoroethyl-amino-furanone is only a minor plant metabolite. However, based on the results of the confined rotational crop study, additional tox studies (acute toxicity testing, genotoxicity testing and subacute rat study) were conducted to show that the tox profile of the metabolite is covered by the endpoints derived from the parent compound. In the *in vitro* genotoxicity package, the Ames and HPRT tests were negative and the chromosome aberration test was positive. Therefore, two *in vivo* studies (*in vivo* micronucleus test and *in vivo* unscheduled DNA synthesis) were conducted, both of which were negative. Based upon the overall findings, BYI 02960-difluoroethyl-amino-furanone can be considered not genotoxic. The acute oral LD_{50} in rats was higher than 2000 mg/kg. The NOAEL of the 28-day rat study was 3000 ppm equating to 243 and 273 mg/kg/day in males and females, respectively based on body weight effects.

Several toxicology studies exist for two plant metabolites BYI 02960-CHMP ((6-chloro-3-pyridyl) methanol) and BYI 02960-6-CAN (6-chloronicotinic acid), which are metabolites common to other pesticides. For BYI 02960-CHMP, the Ames test was negative. The acute oral rat LD_{50} was 1842 mg/kg in males and 1483 mg/kg in females. In a 90-day rat study, BYI 02960-CHMP administered continuously via dietary administration to Sprague Dawley rats (10/sex/group) at concentrations of 160, 800, 4000 and 20 000 ppm resulted in decreases in mean body weights and mean food consumption in both sexes and a statistically significant increases in serum alkaline phosphatase activity in the 20000 ppm group females at study termination only. Histologically, dose-related eosinophilic intranuclear inclusions were seen in the proximal tubular epithelium of kidneys for 20000 ppm males and females and 4000 ppm males. The no observed effects level (NOEL) was 800 ppm (48.9 mg/kg/day) in males, and 4000 ppm (275.9 mg/kg/day) in females. When the NOEL is expressed in BYI 02960 equivalents, it equates to 97.8 and 551.8 mg/kg/day. Therefore, BYI 02960-CHMP was less toxic than BYI 02960 after subchronic administration to the rat.

For BYI 02960-6-CAN (6-chloronicotinic acid) an acute oral rat toxicity study and an Ames test were performed for the registration of acetamiprid. BYI 02960-6-CAN was not genotoxic and not acutely toxic. For the remaining metabolites, BYI 02960-difuranone and BYI 02960-acetic acid, a set of two *in vitro* genotoxicity tests (the Ames assay and the *in vitro* micronucleus assay) were conducted on each metabolite. The results of these two sets of studies were negative.

The results of all metabolite studies are summarized in the following table.

Table 5.11-08: Summary of toxicity studies with the metabolites

Study	Species	Results
-DFA		
Ames test M-409724-01-2	*Salmonella Typh.*	Negative
In vitro HPRT Locus Gene Mutation Assay M-409727-01-2	Chinese hamster V79 lung cells	Negative
In vitro Chromosome Aberration Test M-409726-01-2	Chinese hamster V79 lung cells	Negative
Rat Acute Oral Study M-393372-01-2	Sprague Dawley Rat	300 mg/kg < LD_{50} < 2000 mg/kg
14-day range-finding study M-414152-01-2	Wistar rat	NOAEL = 500 ppm (equating to 51 mg/kg/day) based on clinical chemistry changes
90-day dietary study M-424611-01-2	Wistar rat	NOAEL = 200 ppm (13.7/15.6 mg/kg bw in M/F) Decreased BW and FC, decreased Hg conc., mean corpuscular volume, mean corpuscular Hg, hematocrit in females, decreased Glc, Tbil, increased urea, higher ketones, focal glandular erosion/necrosis
BYI 02960-DFEAF		
Ames test M-409728-01-2	*Salmonella Typh.*	Negative
In vitro HPRT Locus Gene Mutation Assay M-420095-01-2	Chinese hamster V79 lung cells	Negative
In vitro Chromosome Aberration Test M-420108-01-2	Chinese hamster V79 lung cells	Positive in absence of metabolic activation
In vivo tests	**Dose levels**	
Micronucleus Test in male mice – ip administration (M-420540-01-2)	125, 250 and 500 mg/kg	Negative
Unscheduled DNA synthesis_ oral administration M-420111-01-2	1000 and 2000 mg/kg	Negative
Rat Acute Oral Study M-409674-01-2	Sprague Dawley Rat	LD_{50} cut off ≥ 2 000 mg/kg
Range-finding dietary rat study M-426158-01-2	Wistar Rat	Lower blood glucose concentration from 1280 ppm (equating to 135 mg/kg/day) in females
28-day dietary study M-426136-01-2	Wistar Rat	NOAEL = 3000 ppm (243 and 273 mg/kg/day in males and females, respectively) based on body weight effects.

Table 5.11-08: Summary of toxicity studies with the metabolites (cont'd)

Study	Species	Results
BYI 02960-CHMP		
Ames test M-195904-01-2	*Salmonella Typh.*	Negative
Rat Acute Oral Study M-195899-01-2	Sprague Dawley Rat	LD_{50} in males = 1842 mg/kg LD_{50} in females = 1483 mg/kg
90-day dietary study M-195901-01-2	Sprague Dawley Rat	NOEL= 800 ppm (48.9 mg/kg:/day) in males and NOEL = 4000 ppm (275.9 mg/kg/day) in females BWG and FC decreases, increase in alkaline phosphatase activity, eosinophilic intranuclear inclusions in proximal tubular epithelium of kidney
BYI 02960 - 6- CNA		
Ames test M-195932-01-2	*Salmonella Typh.*	Negative
Rat Acute Oral Study M-195930-01-2	Sprague Dawley Rat	LD50 ≥ 5 000 mg/kg/day in both males and females
BYI 02960-amino-furanone		
Ames test [ADD DART hyperlink]	*Salmonella Typh.*	Negative
In vitro micronucleus assay [ADD DART hyperlink]	Human lymphocytes	Negative
BYI 02960-acetic acid		
Ames test [ADD DART hyperlink]	*Salmonella Typh.*	Negative
In vitro micronucleus assay [ADD DART hyperlink]	Human lymphocytes	Negative

- **Conclusions - ADI, AOEL, maximum concentration in drinking water**

➢ Proposed Acceptable Daily Intake (ADI) and Chronic Reference Dose (cRfD)

The potential risk for consumers is mainly linked to chronic exposure to possible residue of BYI 02960 in the food. Therefore, the ADI (cRfD) should be based on the results of the following chronic studies:

Table 5.11-09: Summary of long term studies

Studies (Document N°) (Dose levels in ppm)		NOEL/NOAEL		LOEL		Target organ(s) and treatment-related effects
		ppm	mg/kg/day	ppm	mg/kg/day	
1 year dog study (M-425272-01-2) (0, 150, 300 & 1000)		300	7.8 / 7.8 (M/F)	1000	28.1 / 28.2 (M/F)	Minimal to slight degeneration of skeletal muscle (gastrocnemius and biceps femoris) in both sexes
2 year rat carcinogenicity (M-428257-01-1) (0, 80, 400 & 2000)		400	15.8 / 22.5 (M/F)	2000	80.8 / 120 (M/F)	Target organs: liver & thyroid in either sex; lung in females. No tumours
Mouse oncogenicity (M-425975-01-1) (0, 70, 300 & 1500)		300	43 / 53 (M/F)	1500	224 / 263 (M/F)	Target organs: liver either sex; kidney in males No tumours
Rat Multigeneration (M-417665-01-2) (0, 100, 500 & 1800)	Parent	500 / 100 (M/F)	32.3 / 7.8 (M/F)	1800 / 500 (M/F)	119.8 / 39.2 (M/F)	*Males:* ↑ liver weights (P) ↑ thyroid weights (P) ↑ incidence of centrilobular hypertrophy (minimal - P) *Females:* ↓ BW (premating, gestation, and lactation; F_1) ↓ BWG (premating; P and F_1) ↓ terminal body weights (P & F_1)
	Reprod	500	32.3 / 39.2 (M/F)	1800	119.8 / 140.2 (M/F)	↓ cycle number (F_1), litter size (F_1), and number of implants (F_1)
	Pups	100	7.8 (M/F)	500	39.8 (M/F)	↓ BW and BWG (F_2); with Secondary to ↓ BW organ weight changes in brain, thymus, and spleen

In accordance with internationally accepted procedures the ADI (or Reference Dose RfD) is estimated on the basis of the NOAEL obtained in a chronic toxicity study in the most sensitive species. The lowest NOAEL is in the rat or the dog, which are therefore the most sensitive species. The lowest NOAEL of 7.8 mg/kg/day was observed in both the rat two-generation reproduction study based on body weight effects in the females and in the one year dog study based on histopathological findings in

the skeletal muscles. None of the other toxicological studies showed any indication of mutagenicity; slight reproductive toxicity was only observed at a maternal toxicity dose and no developmental toxicity potential was observed. Therefore, taking into account the toxicological profile of BYI 02960, a margin of safety (MOS) of 100 is considered to be appropriate. Based on these considerations, the following ADI value (= chronic Reference Dose cRfD) is proposed for BYI 02960:

ADI = 7.8 (mg/kg/day)/100 = 0.078 mg /kg/day

- Proposed Acute Dietary Exposure aRfD (Acute reference dose)

The potential acute risk for consumers is mainly linked to single exposure to possible residue of BYI 02960 in the food. Therefore, the aRfD should be based on the results of the following studies:

Table 5.11-10: Summary of relevant short term studies

Studies (Document N°) (Concentrations in mg/kg/day)		NOAEL mg/kg/day	LOAEL mg/kg/day	Target organ(s) and treatment-related effects
Acute neurotoxicity in the rat (M-415408-01-2) 0, 20, 35, 50, 200 and 800		35 (M/F)	50 (M/F)	Piloerection and dilated pupils - At high dose levels: lower muscle tone, rapid respiration, gait incoordination, tremors, reduced motor activity, impaired righting reflex, impaired flexor and tail pinch responses
Rat developmental toxicity (M-363938-01-2) 0,15,50&150	+	15	50	Decreased mean BWG and FC
	Fetuses	50	150	Decreased fetal BW, reduced ossification of a few skull bones
Rat complementary toxicity (M-425810-01-2) 0, 20 & 30	Dams	30	>30	No maternal toxicity
Rabbit developmental toxicity (M-423559-01-1) 0, 7.5, 15 & 40	Dams	15	40	Decreased BW, BWG and FC (GD6-10)
	Fetuses	40	>40	No treatment-related effects

Only slight body weight gain effects were observed at the top dose in the developmental toxicity studies in both rats and rabbits. Acutely toxic effects were seen only in the acute neurotoxixity study, where typical signs of nicotinergic insecticides have been observed. Therefore, it seems appropriate to set up the aRfD using the NOAEL of the acute neurotoxicity study. Taking into account the toxicological profile of BYI 02960, a margin of safety (MOS) of 100 is considered to be appropriate. Based on these considerations, the following aRfD value is proposed for BYI 02960:

aRfD = 35 (mg/kg)/100 = 0.35 mg /kg

- Proposed Acceptable Operator Exposure Level (AOEL)

Since health risks to operators relate to short term rather than to chronic exposure, the NOAELs derived from short term and developmental toxicity studies and the rat 2-generation reproduction study should be taken into account for the purposes of establishing an AOEL for the EU.

BYI 02960 has low acute toxicity to mammals irrespective of the route of exposure (oral, percutaneous or inhalation exposure). It is not a skin sensitizer, it is not irritating to skin and causes only a slight redness of the conjunctivae in the rabbit.
Subchronic studies showed that the liver is a target organ in rodents and dogs. Thyroid effects are limited to rats through a liver enzyme induction mechanism and the kidney is a target organ for both mice and dogs. Furthermore atrophy or degeneration of myofibers of skeletal muscles is observed in dogs. In the rat 2 generation reproduction study, slight decreases in cycle number, litter size and number of implants are observed in the second generation at the top dose where significant body weight effects are observed in the dams. Limited body weight effects are also observed at the intermediate dose in the dams with 20.5% decrease in body weight gain compared to controls in the first generation during premating and 5.9% decrease in body weight or 16.3% decrease in body weight gain in the second generation during premating. Body weight effects during gestation and lactation are seen only in the second generation.
The relevant NOAELs to be considered for calculation of the operator exposures are summarized in the following table.

Table 5.11-11: Summary of relevant data for the calculation of operator exposures

Studies (Document N°) (Dose levels in ppm)		NOEL/NOAEL		LOAEL		Target organ(s) and treatment-related effects
		ppm	mg/kg/day	ppm	mg/kg/day	
90-day dog study (M-369978-01-2) (0, 400, 1200 & 3600/2400)		400	12 / 12 (M/F)	1200	33 / 41 (M/F)	Liver: increased weight in both sexes; brown pigment in Kupffer cells in females (high dose) Kidney: increased relative weights in both sexes Skeletal muscle: myofiber atrophy/ degeneration in both sexes
90-day rat study (M-329048-03-2) (0, 100, 500 & 2500)		500	30 / 38 (M/F)	2500	156 / 186 (M/F)	Liver: centrilobular hepatocellular hypertrophy in both sexes Thyroid: follicular cell hypertrophy in males only
90-day mouse study (M-328668-03-2) (0, 100, 500 & 2500)		500	80.6 / 98.1 (M/F)	2500	407 / 473 (M/F)	Liver: increased diffuse hepatocellular vacuolations Kidney: decreased multifocal/diffuse Corticoepithelial vacuolation
Rat teratology (M-363938-01-2 M-425810-01-2) (0, 15, 20, 30, 50 & 150 mg/kg/day)	Dams	-	30	-	50	Decreased mean BWG and food consumption (FC)
	Foetus	-	50	-	150	Decreased fetal BW; Reduced ossification of a few skull bones
Rabbit teratology (M-423559-01-1) (0, 7.5, 15 & 40 mg/kg/day)	Dams	-	15	-	40	Decreased BW, BWG, corrected BWG, and FC (GD6-10)
	Foetus		40	-	40	No treatment-related effects
Rat Multigeneration (M-417665-01-2) (0, 100, 500, 1800)	Parent	500 / 100 (M/F)	32.3 / 7.8 (M/F)	1800 / 500 (M/F)	119.8 / 39.2 (M/F)	*Male*: Increased liver & thyroid weights (P) Increased incidence of centrilobular hypertrophy (minimal - P) *Female*: Decreased BW (premating, gestation, and lactation; F_1) Decreased BWG (premating; P and F1) Decreased terminal body weights (P & F_1)
	Reprod	500	32.3 / 39.2 (M/F)	1800	119.8 / 140.2 (M/F)	Decreased cycle number (F_1), litter size (F_1), and number of implants (F_1)
	Pups	100	7.8 (M/F)	500	39.8 (M/F)	Decreased BW and BWG (F_2); with Secondary to BW decreases: organ weight changes in brain, thymus, and spleen

From this table, the most sensitive species is the dog with the Lowest Observed Adverse Effect Level (LOAEL) obtained in the 90-day rat study: 33 mg/kg/day, based on myofiber atrophy seen in skeletal muscle. The second lowest LOEL is obtained in the rat 2 generation study: 39.2 mg/kg/day, based on slight body weight effects. Thus, the most sensitive endpoint is seen in the dog. Therefore it is more appropriate to use the 90-day dog study to set up the AOEL.
Available studies indicate that BYI 02960 is well absorbed by rats following oral administration by gavage. Therefore, adjustment for oral absorption is not considered necessary when calculating systemic AOEL for the EU.
Based on this toxicological profile, the current conventional (EU) Uncertainty Factor (UF) of 100 is considered to be appropriate for setting AOEL. For this reason the following systemic AOEL is proposed

$AOEL_{systemic}$ = 12 (mg/kg/day)/100 = 0.12 mg /kg/day

- Proposal for classification and labeling

Study Type	Results	EPA Toxicity Category	OECD Classification
Acute Oral	Mortalities observed at 2000 mg/kg; none at 300 mg/kg	II	IV
Acute Dermal	LD_{50} > 2,000 mg/kg	III	V/ Unclassified
Acute Inhalation (4 h)	LC_{50} at 4 hours > 4671 mg/m³	IV	V/ Unclassified
Primary Eye Irritation	Slight redness of the conjunctivae, reversed within 48 hours	III	V/ Unclassified
Primary Skin Irritation	not a dermal irritant	IV	V/ Unclassified
Dermal Sensitization	not a dermal sensitizer	Not Applicable	V/ Unclassified