Bayer CropScience

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Tier 2 Summary of the Toxicological and Toxicokinetic Studies on the Active Substance for

Spirotetramat (BYI 08330)

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Annex IIA
Section 3, Point 5
Document M

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

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IIA 5 Toxicological/Toxicokinetic Studies on the Active Substance

IIA 5.1 Absorption, distribution, excretion and metabolism in mammals

Executive Summary

Following an oral administration of [azospirodecenyl-3-^{14}C] BYI 08330 to male and female rats at a 3 mg/kg bw dose rate, the gastrointestinal absorption accounted for >90 % of the dose. Excretion was very fast and nearly completed after 24 h. The predominant route of excretion was by urine. No radioactivity was detected in the expired air proving the stability of the labelling position in the molecule. Quantitative whole body autoradiography revealed a fast absorption and distribution of the test compound with peak values observed already 1 h after administration. Among the quantitatively analysed organs, tissues, and fluids, the highest equivalent concentrations were observed in the liver, kidney and blood. Moderate peak concentrations were found in the lung and myocardium, brown fat, skin, the glands and the reproduction organs. Lower concentrations were reached in all other organs and tissues. The lowest peak concentrations were found in the spinal cord, the brain, and the eye. From the peak values, radioactivity concentrations declined by several orders of magnitude below the limit of detection for all organs and tissues within 48 hours in male rats and within 24 hours in female rats.

In the ADME study, four groups of 4 male or female rats were administered by oral gavage with a single dose of [azaspirodecenyl-3-^{14}C]BYI 08330 at a target dose level of 2 and 100 mg/kg body weight. Two groups of 4 male or female rats were pre-treated for 14 days with 2 mg/kg non-radiolabelled BYI 08330 followed by a single radiolabelled dose of 2 mg/kg. The animals of all groups were sacrificed 2 days after dosing. BYI 08330 was very rapidly absorbed from the gastrointestinal tract of male and female rats in all test groups. The absorption commenced immediately after dosing. The absorption rate in the single low dose test was 95 % for male rats and 96 % for female rats. These values refer to the total recovered radioactivity and were calculated from the values of radioactivity in the urine and the body without the gastrointestinal tract. No significant differences in the absorption rate were observed between the single low dose, repeated low dose and single high dose tests.
The maximum of plasma concentration was reached for all dose groups within 0.09 to 2.03 hours after administration (values calculated by pharmacokinetic modelling). From the maximum, the radioactivity concentrations in plasma declined steadily by several orders of magnitude within 48 hours for all dose groups.
Concentrations of radioactivity detected in tissues and organs at the time of sacrifice 48 hours postdose were very low and below the limit of detection for some organs/tissues.
In general, excretion of BYI 08330 residues was very rapid with urine as the predominant excretion route. 48 hours after administration, <0.2 % of the dose were detected in the body and the gastro-intestinal tract. The excretion behaviour was similar for all dose groups.
BYI 08330 was completely metabolized by the rat and no parent compound was detected in the excreta. Identification rates of metabolites were high (87 – 95 % of the dose administered) and only very minor metabolites (<0.7 % of the dose) could not be identified. The main metabolic reaction was cleavage of

the ester group which resulted in the formation of the primary and most predominant metabolite BYI 08330-enol (53 – 87 % of the administered dose). All other identified metabolites could be derived from the enol intermediate. The second prominent metabolic transformation was oxidative demethylation of the 8-methoxy group to BYI 08330-desmethyl-enol (5 – 37 % of the administered dose). Oxidation of the azaspiro moiety to BYI 08330-ketohydroxy and BYI 08330-desmethyl-ketohydroxy were detected as minor pathways. Other very minor metabolic transformations were conjugation of the enol with glucuronic acid to BYI 08330-enol-GA and oxidation of the aromatic methyl group of the enol metabolite to BYI 08330-enol-alcohol.
A sex related difference was observed in the metabolism with male rats showing much higher rates of demethylation to BYI 08330-desmethyl-enol compared to females rats.

Results of the autoradiography and the ADME study were used for a PBPK simulation (physiology based pharmacokinetic modelling). It could be shown, that the experimentally determined ADME behaviour of BYI 08330 in male rats can be well described by PBPK simulations assuming that the compound enters the systemic circulation as the metabolite BYI 08330-enol that is than further metabolized to the BYI 08330-desmethyl-enol. The observed plasma concentrations, their non-linearity in dose, organ concentrations and the metabolization and excretion could be described with very good agreement by simulations based on a common parameterization of the PBPK model. The modelling results indicated the presence of active transport processes for the uptake of BYI 08330 metabolites into liver and kidney and active tubular secretion of BYI 08330 related radioactivity into urine. With a high probability, a certain saturation of the renal transport processes is responsible for the change in shape of plasma concentration curves observed at 100 mg/kg, although the available experimental data did not allow to decide which of both processes, uptake or secretion, is the more relevant one.

In an organ metabolism study, three groups of 4 male rats were administered by oral gavage with a single dose of [azaspirodecenyl-3-^{14}C] BYI 08330 at a target dose level of 2 mg/kg body weight and three other groups at a dose level of 1000 mg/kg body weight. One group of animals from each dose level was sacrificed at 1 h, 8 h, and 24 h after dosage, respectively.
The total radioactivity which included parent compound and metabolites was determined in the excreted urine and faeces samples during the testing time as well as in plasma, liver, kidney, and testis at the time of sacrifice. Investigations on metabolites were performed with urine and plasma samples, and with extracts from liver, kidney, and testis.
The results of the low dose tests matched well with those from the low dose tests in the ADME study. For all time points, the residues in liver and kidney were distinctively higher than in plasma. This finding may indicate that metabolites are transferred by active transport mechanisms from plasma to the excretory organs. The residues in testis, carcass and skin were distinctively lower than in plasma. In plasma and organs, the same metabolites were found as in the excreta but at different proportions. BYI 08330-enol was the most prominent compound in all samples. BYI 08330-desmethyl-enol was found at higher percentages in urine than in plasma and organs. This is probably caused by the more rapid excretion of this more polar metabolite compared to BYI 08330-enol. The highest percentages of BYI 08330-desmethyl-enol in the body were detected in liver, where the compound is formed by metabolic transformation of BYI 08330-enol. The percentages in plasma, kidney and testis were comparable and significantly lower than in liver.

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In high dose tests, the depletion of residues was distinctively slower and the residues in plasma were slightly higher than in liver and kidney. This may be caused by the saturation of the active transport mechanisms resulting in a more uniform distribution of the compound in the body. The residues declined hardly from 1 h to 8 h after dosage and slightly more until 24 h. The residues in testis, carcass and skin were lower than in plasma. The metabolism was similar to the metabolism in low dose tests, with the exception that BYI 08330-desmethyl-enol was found at higher proportions in high dose tests.
In all tests, the first and most important metabolic reaction was the cleavage of the ester bond of the side chain yielding the BYI 08330-enol. The demethylation of the cyclohexyl-O-methyl group to the respective alcohol (BYI 08330-desmethyl-enol) was a further important metabolic reaction as well as the hydroxylation in the azaspiro ring of BYI 08330-enol resulting in BYI 08330-ketohydroxy, which was mainly detected in liver and kidney. Other metabolic reactions like conjugation of the BYI 08330-enol with glucuronic acid, oxidation of one of the methyl groups of the phenyl ring forming the BYI 08330-enol-alcohol, and demethylation of BYI 08330-ketohydroxy to BYI 08330-desmethyl-ketohydroxy were of minor importance. The metabolites detected in plasma and organs were identical with those identified in the excreta of the ADME study.

A second refined PBPK modelling using the results of the ADME and the organ metabolism study predicted a distinct, disproportionate increase of the body burden by BYI 08330-enol and BYI 08330-desmethyl-enol after repeated administration of very high doses of BYI 08330 (dose level >300 mg/kg bw). Overall, the PBPK simulations demonstrated that repeated daily doses of 300 mg BYI 08330/kg bw and higher will lead to non-linear elimination kinetics, resulting in a high body burden in multiple-dose toxicological studies.

A comparative in vitro metabolism study with liver cells from male rats, male mice and male humans revealed differences in the proportions of metabolites formed by the different species.
In the liver cells from all species, BYI 08330 was completely metabolized and no parent compound was detected at the end of the incubation. BYI 08330-enol was the first and most prominent metabolite accounting for 66 – 92 % of total metabolites.
In the rat, the BYI 08330-enol was further metabolised by oxidation reactions to BYI 08330-desmethyl-enol (oxidative demethylation), BYI 08330-enol-alcohol (oxidation of aromatic methyl group) and BYI 08330-ketohydroxy (oxidation of the azaspirodecenyl moiety). Oxidation products accounted for ca. 14 %. Conjugation was not detected as an in vitro metabolic transformation.
In the mouse, oxidative degradation of BYI 08330-enol was detected as a minor “in vitro” metabolic reaction, only (4 % of oxidation products). Conjugation with glucuronic acid to BYI 08330-enol-GA was very prominent with the conjugate accounting for ca. 30 %.
Human liver cells exhibited an “in vitro” metabolism more similar to the one in mouse than in the rat. Conjugation to BYI 08330-enol-GA (6 %) was more prominent than oxidative transformation which was detected in a minor extent (1 %), only.

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The list of metabolites detected in the rat metabolism studies is given in the following table

Report Name	Chemical Structure	IUPAC Name
active substance: BYI 08330 (not detected in animal samples)		**3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate**
BYI 08330-enol		**3-(2,5-dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one**
BYI 08330-enol-GA		**glucuronic acid conjugate of 3-(2,5-dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one**
BYI 08330-desmethyl-enol		**3-(2,5-dimethylphenyl)-4,8-dihydroxy-1-azaspiro[4.5]dec-3-en-2-one**

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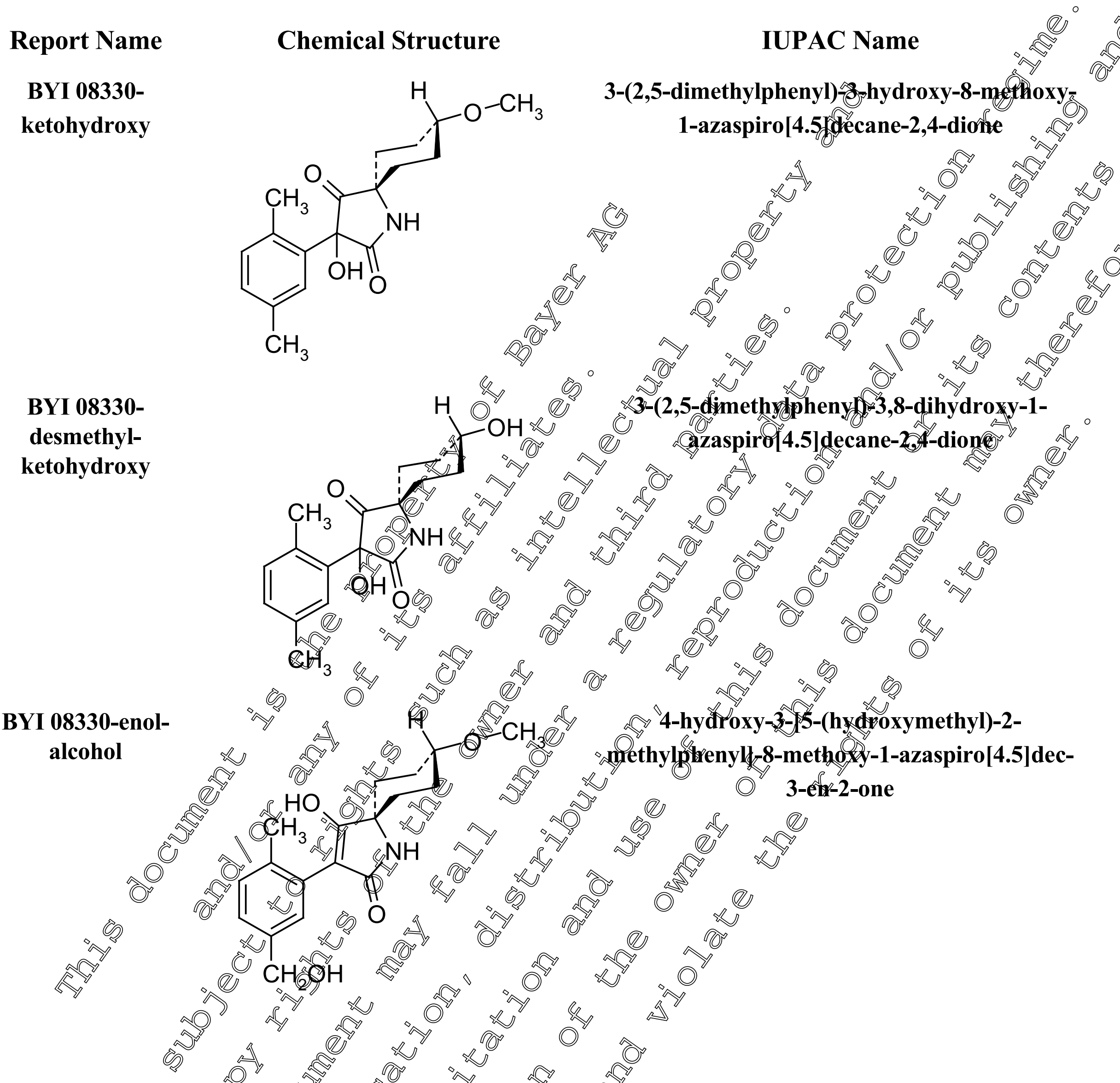
Report Name	Chemical Structure	IUPAC Name
BYI 08330-ketohydroxy		**3-(2,5-dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione**
BYI 08330-desmethyl-ketohydroxy		**3-(2,5-dimethylphenyl)-3,8-dihydroxy-1-azaspiro[4.5]decane-2,4-dione**
BYI 08330-enol-alcohol		**4-hydroxy-3-[5-(hydroxymethyl)-2-methylphenyl]-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one**

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The proposed metabolic pathway of BYI 08330 in the rat is presented in the following figure.

Proposed metabolic pathway of BYI 08330 in male and female rats

BYI08830

BYI08330-enol-alcohol

BYI08330-enol

BYI08330-enol-GA

BYI08330-ketohydroxy

BYI08330-desmethyl-enol

BYI08330-desmethyl-ketohydroxy

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

Four studies and two simulations with BYI 08330 are summarized in this chapter. The first study (Report No. MEF-06/15) reports the distribution and excretion of the radiolabelled test material from the 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, faeces, and air expired.

In the second study (Report No. MEF-048/04), the absorption, distribution, metabolism, and excretion of the radiolabelled test material from male and female rats were tested at a single low dose, at a single high dose and at repeated dose experiments.

The first simulation (Report No. BTS-WSM0602) presents a physiology based pharmacokinetic (PBPK) simulation of BYI 08330 in male rats. Using results from the above described experimental studies the behaviour of BYI 08330 was modelled for low and high dose administration.

In a third experimental study (Report No. MEF-06/328), the metabolism of BYI 08330 in urine, plasma, liver, kidney, and testis of male rats was investigated for the time points 1 h, 4 h, and 24 h after dosing. A single low dose of 2 mg/kg bw and a single very high dose of 1000 mg/kg bw were administered in this study.

The results of this study were used for a refined second PBPK simulation (Report No. BTS-WSM0603-1) modelling of the behaviour of BYI 08330 after repeated very high dose administration.

The fourth study (Report No. SA 05319) described in this chapter is a comparative in vitro metabolism study with liver cells from male rats, male mice, and male humans.

In all studies, the administered test item was ^{14}C-labelled in the azaspirodecenyl-3 position (* denotes the label position):

[azaspirodecenyl-3-^{14}C]BYI 08330

Furthermore, two single low dose toxicokinetic studies with plant metabolites are included in this chapter. These studies were conducted with BYI 08330-enol-glucoside (Report No. MEF-06/006) and BYI 08330-ketohydroxy (Report No. MEF-06/007).

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Report:	**KIIA 5.1.1/01, [redacted] 2006**
Title:	[Azaspirodecenyl-3-^{14}C]BYI 08330: Distribution of the Total Radioactivity in Male and Female Rats Determined by Quantitative Whole Body Autoradiography (QWBA) Including Determination of the Total Radioactivity in Excreta and Exhaled $^{14}CO_2$
Report No & Document No	MEF 06/15 M-269337-01-2
Guidelines:	US EPA Guideline No.: OPPTS 870.7485 Metabolism and Pharmacokinetics, EPA Ref.: 712-C-98-244, August 1998 EU Council Directive 91/414/EEC amended by the Commission Directive 94/79/EC, adopted December 21, 1994 OECD Guideline for Testing Chemicals, 417: Toxicokinetics adopted April 04, 1984 Japanese MAFF New Test Guidelines for Supporting Registration of Chemical Pesticides, 12 Nousan 8147, adopted November 24, 2000, amended June 26, 2001
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] [redacted], Germany Experimental work: 2003-05-09 – 2004-05-24

Executive Summary

The distribution and excretion of BYI 08330 in male and female rats was investigated following a single oral administration of [azaspirodecenyl-3-^{14}C] labelled test item at a non-toxic dose level of 3 mg/kg body weight. Eight rats of each gender were treated and sacrificed 1, 4, 8, 24, 48, 72, 120, and 168 hours after dosing.

BYI 08330 was readily absorbed from the gastrointestinal tract, distributed among almost all organs and tissues, and excreted rapidly within ca. 24 hours (females) and 48 hours (males). Excretion was mainly renal. Only a minor part of ca. 3 – 6 % of the dose was excreted via faeces. Less than 0.01 % of the administered dose was expired as $^{14}CO_2$ or other volatiles during the sampling period of 48 hours. This demonstrates the stability of the azaspirodecenyl-3-^{14}C label with regard to possible formation of volatile products.

Autoradiograms of rat sections were measured by radioluminography. The distribution was followed qualitatively by visual inspection of the autoradiograms. Quantification was performed by measuring the blackening of organs and tissues compared to calibration standards prepared by spiking bovine control blood with different concentrations of a ^{14}C-radiolabelled compound.

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Blackening by radioactivity originating from radiolabelled [azaspirodecenyl-3-^{14}C]BYI 08330 was most intense in the gastrointestinal tract, in the urinary bladder, the kidney, and in the liver. Among the quantitatively analysed organs, tissues, and fluids, the highest equivalent concentrations were observed in the liver, kidney, and blood. Moderate peak concentrations were found in the lung and myocardium, brown fat, skin, the glands, and the reproduction organs. Lower concentrations were reached in all other organs and tissues. The lowest peak concentrations were found in the spinal cord, the brain, and the eye. Peak values of radioactivity were observed already 1 h after administration. From peak values, radioactivity concentrations declined by several orders of magnitude below the limit of detection for all organs and tissues within 48 hours in male rats and within 24 hours in female rats. Only in liver of male rats, very low residues slightly above the limit of quantification were detected 72 hours after dosage. The quantitative data of TRR-values for the organs and tissues in male and female rats are given in table 5.1.1-01 and 5.1.1-02.

I. Material and Methods

A. Material:

1. Test Material:

IUPAC Name:	**cis-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate**
Code name:	**BYI 08330**
Common name:	**Spirotetramat (proposed ISO)**
Empirical formula:	**$C_{21}H_{27}NO_5$**
Molar mass:	**373.45 g/mol**
Water solubility:	**pH 4 = 33.5 mg/L, pH 7 = 29.9 mg/L pH 9 = 19.1 mg/L (unstable) all at 20°C**
n-Octanol/water partition coefficient:	**pH 4 = 2.51, pH 7 = 2.51, pH 9 = 2.50**
Labelling:	**azaspirodecenyl-3-^{14}C**
Specific radioactivity of the original radiolabelled batches:	**3.71 MBq/mg (100.2 µCi/mg)**
Specific radioactivity used for administration:	**3.71 MBq/mg (100.2 µCi/mg)**
Radiochemical purity:	**> 98 % (certified, HPLC and TLC with radiodetection)**
Dose level:	**3 mg/kg body weight**
Vehicle:	**0.5 % aqueous tragacanth suspension**
Stability of the test material:	**The stability of [azaspirodecenyl-3-^{14}C]BYI 08330 was demonstrated by radio HPLC analysis of the administration suspensions of each test immediately after dosing**

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2. Test Animals:

Species:	**Rat (*Rattus norvegicus domesticus*)**
Strain:	**Wistar Hsd/Cpb: WU**
Breeding facility:	**[redacted], Germany**
Sex and numbers involved:	**Males: 8 + 1 control animal** **Females: 8 + 1 control animal** **control animals were dosed with non radiolabelled test item**
Age:	**8 weeks (male rats) and 11 – 12 weeks (female rats) at the time of delivery**
Body weight:	**Males: 202 – 217 g at the time of administration** **200 – 244 g at the time of sacrifice** **Females: 194 – 212 g at the time of administration** **192 – 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 item individually in Makrolon® metabolism cages under conventional hygienic conditions in air-conditioned rooms** **Temperature 20 - 24 °C, relative humidity 33 – 63 %.** **12 / 12 hours light / dark cycle, air change 10 – 15 times per hour.**
Feed and water:	**rat/mice maintenance long life /$z/ (no. 3883.0.15), supplied by [redacted], 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***

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B. Study Design

Dosing

Each of the rats (8 rats per gender) orally received 3 mg/kg bw of [azaspirodecenyl-3-^{14}C]BYI 08330 suspended in 0.5 % aqueous tragacanth after ca. 16 h of fasting. A control animal of each gender was treated with 3 mg/kg bw non-radiolabelled BYI 08330. The dosing suspensions (0.3 mg/mL) were prepared one day before dosing and stored below 4°C. The samples were adjusted to pH 4 to avoid hydrolysis of the test item.

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 administered radioactivity amounts by the specific radioactivity. The actual mean administered dose of [azaspirodecenyl-3-^{14}C]BYI 08330 was 2.63 mg/kg bw for male rats and 2.73 mg/kg bw for female rats. The stability of the test compound in the suspensions was assured by radio HPLC analysis of aliquots after administration.

Collection of excreta

After administration of the radiolabelled test item, the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine, faeces, and the expired air. Urine was collected separately for each animal in a cryogenic trap cooled with dry ice in intervals of 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 (freeze-dried), weighed, and homogenised. The radioactivity was determined by combustion/LSC.

Trapping of expired air

Carbon dioxide and other volatiles from expired air were collected from four male animals and three 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.

Sacrifice and preparation of carcass for autoradiography

One animal of each gender was sacrificed using carbon dioxide 1, 4, 8, 24, 48, 72, 120, and 168 h post administration, respectively. One female rat had to be sacrificed prematurely due to bad health condition. This animal was excluded from the study. The control animals were sacrificed 4 h after dosing.

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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).

Autoradiography

Sagittal sections of 50 µm were cut at ca. -25°C using a cryomicrotome, attached to adhesive tape, and lyophilized overnight in the cooling cabinet of the microtome. Four to five sections showing the relevant organs and tissues were prepared from each animal. Two imaging plates were exposed to the sections at time ranges from 48 to 120 hours. The exposed imaging plates were scanned by laser using the Fuji BAS 5000® image analyser. The sections were stored at about -20°C at all times except during exposure, which was performed at room temperature.

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 distribution of radioactivity concentrations in different organs and tissues. The autoradiograms were quantitatively evaluated using the Tina® software (Raytest, version 2.10 g). Defined areas were set and integrated in each organ or tissue or partial structure 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 7 calibration standards were prepared by spiking bovine control blood with different concentrations of a ^{14}C-radiolabelled compound. The concentrations covered a range from approx. 1,000 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 activity 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.

Radioactivity measurement

The measurement of the 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 only exception was the measurement of radioactivity in the expired air samples for which all reasonable dpm-values were quantified. 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 equal to 12 – 30 dpm) and it was automatically subtracted from the

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measuring 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 the new insecticide BYI 08330 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 (male rats) and 5 (female rats) days following a single oral administration of radiolabelled [azaspirodecenyl-3-^{14}C]BYI 08330 at a dose level of 3 mg/kg body weight (bw). Eight male rats and 7 female rats were treated and sacrificed 1, 4, 8, 24, 48, 72, 120, and 168 (male only) hours after dosing.

Blackening by radioactivity originated from radiolabelled [azaspirodecenyl-3-^{14}C]BYI 08330 was most intense in the gastrointestinal tract, in the urinary bladder, the kidney and in the liver. Among the quantitatively analysed organs, tissues, and fluids, the highest equivalent concentrations were observed in the liver, kidney and blood. Moderate peak concentrations lower than in blood were found in the lung and myocardium, brown fat, skin, the glands and the reproduction organs. Lower concentrations were reached in all other organs and tissues. The lowest peak concentrations were found in the spinal cord, the brain, and the eye.

Peak concentrations of radioactivity were already reached after 1 hour. From the peak values, a continuous decrease of radioactivity concentrations by several orders of magnitude, below the limit of detection was observed for all organs and tissues within 48 hours in male rats and within 24 hours in female rats. Only in liver of male rats, very low residues slightly above the limit of quantification were detected 72 hours after dosage. All quantitative results are presented in Table 5.1.1-01 (male rats) and Table 5.1.1-02 (female rats).

B. Excretion

BYI 08330 was excreted rapidly and completely within ca. 48 hours. Excretion was mainly renal. Only a minor part of ca. 3 – 6 % of the dose was excreted via faeces. 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 0.01 % of the administered dose was expired during the sampling period. This demonstrates the stability of the azaspirodecenyl-3-^{14}C label with regard to possible formation of volatile products. The excretion behaviour is summarized in Table 5.1.1-03 (male rats) and Table 5.1.1-04 (female rats).

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III. Conclusions

The new insecticide BYI 08330 was readily absorbed from the gastrointestinal tract, distributed among almost all organs and tissues, and excreted rapidly mainly via the renal route.
Concentrations of radioactivity were most intense in the gastrointestinal tract, in the urinary bladder, the kidney, and in the liver. The peak value of radioactivity concentrations was reached 1 h after dosing followed by a continuous decrease by several orders of magnitude, below the limit of detection for all organs and tissues within 24 hours in female rats and within 48 hours in male rats. Any retention or accumulation of BYI 08330 related radioactivity in the animal body could be excluded.

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Table 5.1.1-01: Distribution of radioactivity in organs and tissues of male rats after a single oral administration of 3 mg [azaspirodecenyl-3-^{14}C]BYI 08330/kg bw

	Total radioactive residues (TRR) in organs and tissues of male rats [µg a.s. equiv./g wet]							
	Time of sacrifice (hours after administration)							
Organ/ Tissue	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Blood	2.711	1.285	0.277	< LOQ	---	---	---	---
Liver	7.437	5.439	1.173	0.017	< LOQ	0.006	< LOQ	< LOD
Renal cortex	10.635	4.811	1.429	0.008	< LOD	---	---	---
Renal medulla	12.723	7.614	2.391	0.008	< LOD	---	---	---
Brown fat	1.247	0.626	0.177	---	---	---	---	---
Perirenal fat	---	0.076	0.043	< LOD	---	---	---	---
Skeleton muscle	0.658	0.331	0.064	---	---	---	---	---
Myocardium	1.611	0.738	0.177	< LOD	---	---	---	---
Lung	1.097	0.501	0.196	< LOD	---	---	---	---
Spleen	0.537	0.255	0.065	< LOD	---	---	---	---
Pancreas	0.591	0.306	0.068	< LOD	---	---	---	---
Bone marrow	0.681	0.298	0.088	---	---	---	---	---
Testes	0.509	0.404	0.088	< LOQ	---	---	---	---
Brain	0.102	0.056	0.012	< LOD	---	---	---	---
Spinal cord	0.106	0.058	0.011	---	---	---	---	---
Pituitary gland	0.966	0.599	0.101	---	---	---	---	---
Pineal body	1.023	0.479	0.106	---	---	---	---	---
Adrenal gland	2.005	0.683	0.168	---	---	---	---	---
Thymus	0.579	0.236	0.053	< LOD	---	---	---	---
Thyroid gland	1.412	0.639	0.142	---	---	---	---	---
Salivary gland	1.222	0.506	0.100	---	---	---	---	---
Nasal mucosa	0.439	0.319	0.061	---	---	---	---	---
Skin	0.955	0.572	0.106	---	---	---	---	---
Vitreal body (eye)	0.146	0.037	0.024	---	---	---	---	---

--- organ or tissue was visible in the rat sections but not discernible in the radioluminograms
< LOQ = below limit of quantification
< LOD = below limit of detection

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Table 5.1.1-02: Distribution of radioactivity in organs and tissues of <u>female</u> rats after a single oral administration of 3 mg [azaspirodecenyl-3-^{14}C]BYI 08330/kg bw

	Total radioactive residues (TRR) in organs and tissues of female rats [µg a.s. equiv./g wet]						
	Time of sacrifice (hours after administration)						
Organ/ Tissue	1	4	8	24	48	72	120
Blood	1.195	0.365	0.088	---	---	---	---
Liver	4.497	1.318	0.397	< LOD	---	---	---
Renal cortex	5.148	1.489	0.438	---	---	---	---
Renal medulla	7.306	2.624	0.913	---	---	---	---
Brown fat	0.530	0.116	0.037	---	---	---	---
Perirenal fat	0.108	0.053	0.010	---	---	---	---
Skeleton muscle	0.247	0.070	0.018	---	---	---	---
Myocardium	0.749	0.198	0.052	---	---	---	---
Lung	0.823	0.116	0.043	---	---	---	---
Spleen	0.241	0.068	0.017	---	---	---	---
Pancreas	0.281	0.074	0.021	---	---	---	---
Bone marrow	0.268	0.089	0.020	---	---	---	---
Ovary	0.595	0.111	0.028	---	---	---	---
Uterus	0.759	0.170	0.045	---	---	---	---
Brain	0.047	0.013	< LOQ	---	---	---	---
Spinal cord	0.051	0.014	< LOQ	---	---	---	---
Pituitary gland	0.476	0.134	0.032	---	---	---	---
Pineal body	0.504	0.122	0.038	---	---	---	---
Adrenal gland	0.877	0.216	0.065	---	---	---	---
Thymus	0.214	0.066	0.015	---	---	---	---
Thyroid gland	0.567	0.162	0.036	---	---	---	---
Salivary gland	0.578	0.147	0.031	---	---	---	---
Nasal mucosa	0.172	0.074	0.014	---	---	---	---
Skin	0.564	0.140	0.032	---	---	---	---
Vitreal body (eye)	0.060	0.040	0.009	---	---	---	---

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

< LOQ = below limit of quantification

< LOD = below limit of detection

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Table 5.1.1-03: Excretion of radioactivity in urine, faeces and expired air of male rats after a single oral administration of 3 mg [azaspirodecenyl-3-^{14}C]BYI 08330/kg bw

	Radioactivity in percent of dose administered							
Time of sacrifice after admin.	**1 h**	**4 h**	**8 h**	**24 h**	**48 h**	**72 h**	**120 h**	**168 h**
Time after admin.								
Expired air								
24 h					0.003	0.002	0.001	0.002
48 h					0.001	0.001	0.001	0.001
Urine								
1 h	5.17							
4 h		9.07			47.36	51.32	41.79	32.03
8 h			80.63	79.40	19.56	23.91	35.12	36.04
24 h				19.63	30.69	19.44	31.02	38.36
48 h					0.33	0.60	0.77	0.39
72 h						0.14	0.20	0.20
96 h							0.09	0.05
120 h							0.09	0.03
144 h								0.05
168 h								0.02
Faeces								
24 h				7.11	4.56	5.44	6.84	5.38
48 h					0.22	0.23	0.34	0.27
72 h						0.04	0.04	0.03
96 h							0.01	0.01
120 h							0.01	0.01
144 h								0.01
168 h								0.01
Urine + faeces + expired air								
1 h	5.17							
4 h		9.07			47.36	51.32	41.79	32.03
8 h			80.63	79.40	19.56	23.91	35.12	36.04
24 h				26.74	35.25	24.88	37.86	43.74
48 h					0.55	0.83	1.11	0.66
72 h						0.18	0.24	0.23
96 h							0.10	0.06
120 h							0.10	0.04
144 h								0.06
168 h								0.03
Total excreted	**5.17**	**9.07**	**80.63**	**106.13**	**102.73**	**101.11**	**116.33**	**112.90**

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Table 5.1.1-04: Excretion of radioactivity in urine, faeces and expired air of female rats after a single oral administration of 3 mg [azaspirodecenyl-3-^{14}C]BYI 08330/kg bw

	Radioactivity in percent of dose administered						
Time of sacrifice after admin.	**1 h**	**4 h**	**8 h**	**24 h**	**48 h**	**72 h**	**120 h**
Time after admin.							
Expired air							
24 h					0.001	0.002	0.002
48 h					0.001	0.001	0.001
Urine							
1 h	39.55						
4 h		33.76			81.32	25.33	74.29
8 h			83.61	73.58	6.72	54.50	19.99
24 h				19.19	5.69	11.92	5.98
48 h					0.39	0.72	0.45
72 h						0.28	0.08
96 h							0.04
120 h							0.05
144 h							
168 h							
Faeces							
24 h				1.80	1.72	2.21	2.84
48 h					0.29	0.09	0.08
72 h						0.02	0.01
96 h							0.01
120 h							0.01
144 h							
168 h							
Urine + faeces + expired air							
1 h	39.55						
4 h		33.76			81.32	25.33	74.29
8 h			83.61	73.58	6.72	54.50	19.99
24 h				20.99	7.41	14.13	8.82
48 h					0.68	0.81	0.53
72 h						0.30	0.09
96 h							0.05
120 h							0.06
144 h							
168 h							
Total excreted	**39.55**	**33.76**	**83.61**	**94.57**	**96.13**	**95.08**	**103.83**

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Report:	**KIIA 5.1.1/02, 2006**
Title:	[Azaspirodecenyl-3-^{14}C]BYI 08330: Adsorption, Distribution, Excretion and Metabolism in the Rat
Report No & Document No	MEF 048/04 M-268709-02-2
Guidelines:	US EPA Guideline No.: OPPTS 870.7485 Metabolism and Pharmacokinetics, EPA Ref.: 712-C-98-244, August 1998 EU Council Directive 91/414/EEC amended by the Commission Directive 94/79/EC, adopted December 21, 1994 OECD Guideline for Testing Chemicals, 417: Toxicokinetics adopted April 04, 1984 Japanese MAFF New Test Guidelines for Supporting Registration of Chemical Pesticides, 12 Nousan 8147, adopted November 24, 2000, amended June 26, 2001
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	Germany Experimental work: 2003-05-27 – 2005-03-04

Executive Summary

Four groups of 4 male or female rats were administered by oral gavage with a single dose of [azaspirodecenyl-3-^{14}C]BYI 08330 in aqueous Tragacanth® at a target dose level of 2 and 100 mg/kg body weight. Two groups of 4 male or female rats were pre-treated for 14 days with 2 mg/kg non-radiolabelled BYI 08330 followed by a single radiolabelled dose of 2 mg/kg. The animals of all groups were sacrificed 2 days after dosing. The total radioactivity which included the test item and metabolites was determined in plasma samples, the excreta (urine and faeces) as well as in organs and tissues. The metabolism was investigated by radio-HPLC, normal phase TLC and spectroscopic methods in selected urine samples and faeces extracts.

Between 91.4 % and 99.8 % of the administered dose were recovered from measurement of the total radioactivity in urine and faeces as well as in organs and tissues at sacrifice.

[Azaspirodecenyl-3-^{14}C]BYI 08330 was very rapidly absorbed from the gastrointestinal tract of male and female rats in all test groups. The absorption was commenced immediately after dosing. In this study, the absorption rate in all tests was between 89 and 98 % of the <u>total recovered radioactivity</u>, calculated from the values of the urine and the body without the gastrointestinal tract. No significant differences in the absorption rate were observed between the low, high dose, and repeated dose tests. The absorption rate in the single low dose test was 95 % for male rats and 96 % for female rats.

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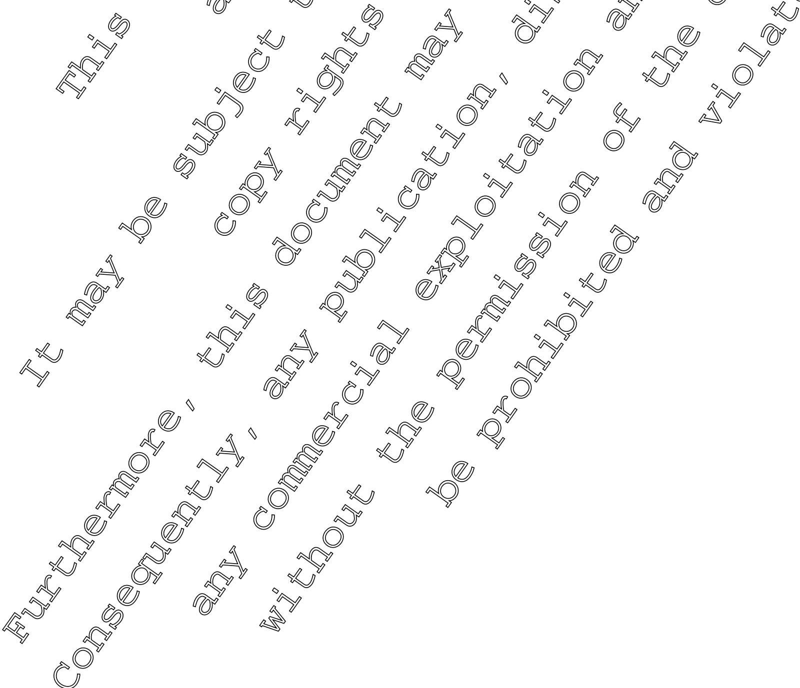
The maximum of plasma concentration was reached for all dose groups within 0.09 to 2.03 hours after administration (values calculated by pharmacokinetic modelling). The radioactivity concentrations in plasma declined steadily by several orders of magnitude from the maximum within 48 hours for all dose groups.
Concentrations of radioactivity detected in tissues and organs at the time of sacrifice 48 hours postdose were very low and below the limit of detection for some organs/tissues.
In general, excretion of BYI 08330 residues was very rapid in urine being the predominant excretion route. Faecal excretion only accounted for 2 to 11 % of the dose in male and female rats. 48 hours after administration, <0.2 % of the dose were detected in the body and the gastro-intestinal tract. The excretion behaviour was similar for all dose groups.

BYI 08330 was completely metabolized by the rat and no parent compound was detected in the excreta. Identification rates of metabolites were high (87 – 95 % of the administered dose) and only very minor metabolites (<0.7 % of the dose) could not be identified. The main metabolic reaction was cleavage of the ester group which resulted in the formation of the primary and most predominant metabolite BYI 08330-enol (53 – 87 % of the administered dose). All other identified metabolites could be derived from the enol intermediate. The second prominent metabolic transformation was oxidative demethylation of the 8-methoxy group to BYI 08330-desmethyl-enol (5 – 37 % of the administered dose). Oxidation of the azaspiro moiety to BYI 08330-ketohydroxy and BYI 08330-desmethyl-ketohydroxy were detected as minor pathways. Other minor metabolic transformations were conjugation of the enol with glucuronic acid to BYI 08330-enol-GA and oxidation of the aromatic methyl group of the enol metabolite to BYI 08330-enol-alcohol.
A sex related difference was observed in metabolism with male rats showing much higher rates of demethylation to BYI 08330-desmethyl-enol compared to females rats.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I. Material and Methods

A. Material

1. Test Material

IUPAC Name:	**cis-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate**
Code name:	**BYI 08330**
Common name:	**Spirotetramat (proposed ISO)**
Empirical formula:	**$C_{21}H_{27}NO_5$**
Molar mass:	**373.45 g/mol**
Water solubility:	**pH 4 = 33.5 mg/L, pH 7 = 29.9 mg/L pH 9 = 19.1 mg/L (unstable) all at 20°C**
n-Octanol/water partition coefficient:	**pH 4 = 2.51, pH 7 = 2.51, pH 9 = 2.50**
Labelling;	**azaspirodecenyl-3-^{14}C**
Specific radioactivity of the original radiolabelled batches:	**test 2 – 5: 3.71 MBq/mg (100.2 µCi/mg) test 8 + 9: 3,67 MBq/mg (99.1 µCi/mg)**
Specific radioactivity used for administration:	**test 2 + 5: 3.71 MBq/mg (100.2 µCi/mg) test 3 + 4: 0.07 MBq/mg (1.9 µCi/mg) test 8 + 9: 3.67 MBq/mg (99.1 µCi/mg)**
Radiochemical purity:	**> 98 % or > 99 % (certified, HPLC and TLC with radiodetection)**
Dose level:	**test 2 + 8: 2 mg/kg body weight test 3 + 4: 100 mg/kg body weight test 5 + 9: 2 mg/kg body weight, animals pre-treated for 14 days with 2 mg/kg bw non-radiolabelled BYI 08330**
Vehicle	**0.5 % aqueous tragacanth suspension**
Stability of the test material	**The stability of [azaspirodecenyl-3-^{14}C]BYI 08330 was demonstrated by radio HPLC analysis of the administration suspensions of each test immediately after dosing**

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2. Test Animals

Species:	**Rat (*Rattus norvegicus domesticus*)**
Strain:	**Wistar Hsd/Cpb: WU**
Breeding facility:	**, Germany**
Sex and numbers involved:	**Males: 12 animals (3 groups with 4 animals)** **Females: 12 animals (3 groups with 4 animals)**
Age:	**9 – 10 weeks (male rats) and 12 – 13 weeks (female rats) at the time of delivery**
Body weight:	**Males: 200 – 216 g at the time of administration** **204 – 246 g at the time of sacrifice** **Females: 197 – 212 g at the time of administration** **197 – 212 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 22 - 26 °C, relative humidity 37 – 76 %.** **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 , 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***

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B. Study Design

Dosing

Each of the rats (4 animals per group) received orally 2 or 100 mg/kg bw of azaspirodecenyl-3-^{14}C labelled BYI 08330 suspended in 0.5 % aqueous tragacanth after 16 h fasting. The dosing suspensions (0.2 or 10 mg /mL for the low and high dose level) were prepared one day before dosing and stored at 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). Actual administered doses were measured by LSC assay of aliquots of the administration suspensions. The actual mean administered doses of [azaspirodecenyl-3-^{14}C]BYI 08330 were in the range of 1.65 - 2.02 mg/kg bw for the low dose tests and 84.9 - 92.7 mg/kg bw for the high dose tests. The stability of the test compound in the suspensions was assured by radio HPLC analysis of aliquots after administration.

Collection of blood

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 closed with adhesive tape. The capillaries were centrifuged at ca. 12000 g for about 10 minutes using a hematocrit centrifuge to separate the plasma from the formed constituents (mainly erythrocytes). After centrifugation, the capillary was broken at the border between the plasma and the formed constituents and the plasma (ca. 30 µL) was placed onto a small metal dish for weighing. The dish was then placed into a scintillation vial for radioactivity measurement

Collection of excreta

Urine was collected at various times separately for each animal in a cryogenic trap cooled with dry ice. 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. Urine samples were stored in a freezer before analysis.

Faeces were collected at various times separately for each animal in a cryogenic trap before they were lyophilised (freeze-dried), weighed, and homogenised. The radioactivity was determined by combustion/LSC. Lyophilised samples were stored at room temperature before extraction/analysis.

Sacrifice and organ/tissue sampling

At the respective test end (48 h after administration), the animals were anaesthetised using Pentobarbital-Na. They were sacrificed by transection of the cervical blood vessels. After transection of the cervical blood vessels, the oozed out blood was collected into test tubes coated with heparin and was separated afterwards into plasma and erythrocytes by centrifugation. The following organs/tissues were collected: erythrocytes, plasma, spleen, gastro-intestinal tract (GIT), liver, kidneys, perirenal fat, adrenal gland, testis (male only), ovaries (female only), uterus (female only), skeleton muscle, femur bone, heart, lung, brain, thyroid gland, skin, and carcass.

The organs and tissues prepared at the end of the experiments were weighed immediately after the dissection and again after lyophilisation. Finally, they were homogenised and the radioactivity was determined by combustion/LSC. The radioactivity remaining in the GIT, skin, and residual carcass was determined by combustion/LSC in order to establish the radioactivity balance. For the small organs and

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tissues (e.g. renal fat, uterus, adrenal glands, thyroid, and ovaries), only the wet weight was weighed and the samples then solubilised with BTS 450® (Beckman Tissue Solubiliser).

Radioactivity measurement

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). For this purpose, the volume of the entire solution was determined and radioactivity of an aliquot (1 – 3 replicates) of the solution was measured using the following scintillation counters:
Small organs or tissues were solubilised by means of a tissue solubiliser (e.g. BTS-450®). The solubilised samples or aliquots of them were acidified with hydrochloric acid or glacial acetic acid and mixed with a suitable scintillation cocktail (e.g. Quickszint 401, Zinsser Analytic GmbH). The radioactivity was then measured in a scintillation counter. This method was used for the following test materials: renal fat, adrenal glands, thyroid, ovaries, and uterus.
Solid samples (1 – 5 aliquots) were weighed and combusted in an oxygen atmosphere using the following equipments Oxidizer 307/387 (Packard Instruments) for combustion of faeces, erythrocytes, lyophilised organs and tissues like e.g. spleen, liver, lung, bone muscle, gastrointestinal tract (GIT), residual carcass and solids (e.g. from faeces after extraction). Carbosorb E (9 mL) was used to trap the combustion product CO_2 and Permafluor E+ (10 mL; Packard Instruments) as scintillator for LSC.

Metabolite analysis

Urine samples were analysed without sample preparation. Freeze-dried faeces samples of all animals from 0 - 24 h post administration were combined for each test. The radioactivity in each sample material used for analysis was determined by combustion/LSC. Each composite faeces sample was extracted successively 3 x with acetonitrile / water (8: 2, v/v) using a Polytron homogeniser. Each extract was collected by centrifugation and its volume was measured. Aliquots of each extract were radioassayed by LSC. The three extracts of each faeces preparation were combined and concentrated to the aqueous remainder and partitioned 3 times against n-hexane. Aliquots of aqueous and organic phases were radioassayed by LSC. The aqueous phase was concentrated and used for HPLC analysis.
The metabolites in the samples were quantified by HPLC using a reversed phase column (C18) and the eluting solvents water/formic acid = 99: 1 (v/v) and acetonitrile/water/formic acid = 97: 2: 1 (v/v/v) in the gradient mode. Detection was performed by a UV (254 nm) and a radioisotope detector with a glass scintillator.
In order to check the completeness of the chromatographic elution, several representative samples were injected, re-collected, and radioassayed by LSC. The column recoveries were between >99 and 101 % of the injected amount of radioactivity.

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The first method for identification of metabolites in urine and extracts of faeces was HPLC co-chromatography with radiolabelled and non-radiolabelled reference items.
One-dimensional TLC on silica 60 F254 (normal phase) plates was used as the second method for co-chromatographic identification of metabolites. The plates were developed over a separation path of ca. 16 cm in a standard TLC-chamber.
The three following TLC solvent systems were used:

System 1: Ethyl acetate/propanol-2/water (65/23/12 v/v/v), with chamber saturation

System 2 Dichloromethane/methanol/ammonia solution 25 % (90/10/1 v/v/v), with chamber saturation

System 3 Trichloromethane/methanol/glacial acetic acid/water (40/10/2/1 v/v/v/v), with chamber saturation

For the TLC confirmation of metabolites, aliquots of urine and faeces extracts were fractionated with the HPLC profiling method. The collected peaks were then concentrated to the aqueous remainder. Aliquots of these samples and of the solutions of the reference items were applied individually and overlapped with each other on the TLC-plates. Co-chromatographic correspondence was assessed either by visual inspection of the plate under UV light at 254 nm and its associated radioluminogram or by analysis of the radioluminogram only, in cases where radioactive reference items were used.
In addition to co-chromatography, the two main urine metabolites were identified by LC-MS and LC-MS/MS of isolated and purified fractions.

II. Results and Discussion

A. Absorption

For all tests, the mean equivalent concentrations (C) of total radioactivity from four animals were used to calculate the basic toxicokinetic parameters from plasma curve analysis. The pharmacokinetic software TOPFIT (version 2.0) was used with a 3-compartment disposition model for fitting of the data. Correlation coefficients were excellent for all tests (0.994 – 1.00).
BYI 08330 was very rapidly absorbed from the gastrointestinal tract of male and female rats in all test groups. The absorption commenced immediately after oral dosing. Values calculated for the absorption half-lives were in the range of 0.6 – 10 min. No significant differences were observed between the low dose, high dose, and repeated dose tests. BYI 08330 was completely absorbed even at the high dose level of 100 mg/kg bw since 89 - 98 % of the total recovered radioactivity were excreted via the renal route. Between 91 – 100 % of the administered radioactivity were recovered in urine, faeces, and the body of animals. The balance of radioactivity for all tests is presented in table 5.1.1-05.

B. Distribution

Radioactivity in plasma of male and female rats following a single administration of 2 mg/kg bw reached its calculated maximum at 0.9 and 0.1 hr postdose, respectively. The calculated maximum radioactivity concentrations in plasma were 4.41 and 4.15 mg eq./kg bw, in males and females, respectively. These peak concentrations amounted to ca. 2 times of the equal distribution concentration of BYI 08330. After reaching the maximum, females showed slightly lower decline of plasma radioactivity concentration as compared with males.
Absorption of the radioactivity was slightly slower for males compared to females. Absorption was followed by a fast initial elimination phase and a moderate terminal elimination phase. The area under

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the curves ($AUC_{(0-\infty)}$) indicated a slightly higher systemic exposure for males than for females. The elimination rate constant was very similar for males and females. The mean residence time was low in males and slightly higher in females.

The results obtained from the repeated low dose experiments with pre-treated animals were in the same range as those from the single low dose tests. Absorption of the radioactivity was similar for males and females and was followed by a fast initial elimination phase and a moderate terminal elimination phase. The mean residence time was low and similar for males and females.

The results from the high dose experiments indicated a sex difference in the maximum plasma concentration. The C_{max} – value was significantly higher for males than for females. Compared to all low dose tests and taking the AUC – values into account, the plasma concentrations were more or less proportional to the dose ratio. This indicated that the absorption process had not been (over)saturated at the high dose level of 100 mg/kg bw. The absorption of the radioactivity was slightly slower than in the low dose experiments and slightly slower for the males compared to females. As in low dose tests, the absorption was followed by a fast initial elimination phase and a moderate terminal elimination phase. The area under the curves ($AUC_{(0-\infty)}$) indicated a slightly higher systemic exposure for males than for females. The mean residence time was in the same range as in low dose test and quite similar for males and females. Comparison of the absorption phases of the kinetic curves between the low and high dose groups showed a broader maximum for males of the high dose test. The maximum concentration (C_{max}) was reached slightly later at this dose level and the following initial elimination phase was slightly longer than observed for the low dose level. The curves at the end of the terminal elimination phase were comparable. Radioactivity levels in plasma are summarised in table 5.1.1.-06. The calculated pharmacokinetic parameters are shown in table 5.1.1-07.

The distribution of radioactive residues in the body was analyzed at the time of sacrifice 48 h after dosing by radioactivity measurement of the major organs and tissues. Less than 0.2 % of the administered dose was detected in the body.
The residues in all organs and tissues at the time of sacrifice were low in all tests and partly below the limit of detection. The highest equivalent concentrations were detected in the liver (0.002 – 0.18 mg/kg) and kidney (0.001 – 0.11 mg/kg), the responsible organs for the degradation and excretion. The distribution of radioactivity levels in organs and tissues of male and female rats 48 h after oral administration of [azaspirodecenyl-3-^{14}C]BYI 08330 is shown in Table 5.1.1-08.

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C. Metabolism

No parent compound was detected in urine and faeces of all tests.

The main metabolic reaction was cleavage of the ester group which resulted in the formation of the primary and most predominant metabolite BYI 08330-enol (53 – 87 % of the administered dose). All other identified metabolites could be derived from the enol intermediate. Further metabolic transformations were oxidative demethylation of the 8-methoxy group to BYI 08330-desmethyl-enol (5 – 37 % of the administered dose) and oxidation of the azaspiro moiety to BYI 08330-ketohydroxy (0.5 – 1.1 %) and BYI 08330-desmethyl-ketohydroxy (0.1 – 0.7 %). Other minor metabolic transformations were conjugation of the enol with glucuronic acid to BYI 08330-enol-GA (0.2 – 0.8 %) and oxidation of the aromatic methyl group of the enol metabolite to BYI 08330-enol-alcohol (0.4 – 1.6 %). A sex related difference was observed in metabolism. Male rats exhibited much higher rates of demethylation of BYI 08330-enol to BYI 08330-desmethyl-enol (25 – 37 %) compared to females rats (5 – 10 %). Demethylation was more pronounced in the high dose tests than in the low dose tests.

Identification rates were high in the range of 87 to 95 % of the administered dose. Only 0.4 – 3.5 % in total could not be identified. No single unidentified metabolite accounted for >0.7 % of the dose. The proportion of non identified metabolites was higher for male rats than for female rats.

Metabolites excreted with urine and faeces following oral administration of [azaspirodecenyl-3-^{14}C] BYI 08330 are shown in Table 5.1.1-10. The proposed metabolic pathway of BYI 08330 in the rat is presented in Figure 5.1.1-01

D. Excretion

The excretion of BYI 08330 was fast and almost completed 24 h after administration. Significant sex or dose related differences were not observed. Excretion was mainly renal and quite similar for males and females in low, high and repeated low dose tests. About 88 % to 95 % of the administered dose was eliminated via urine and about 2 % to 11 % via faeces. In every dose group, quantitative recovery of dosed radioactivity (91.44 – 99.78 % of dose) was accomplished.

The formation of $^{14}CO_2$ and volatile metabolites was investigated for male and female rats in the autoradiography study (see 5.1.1/01 and Tables 5.1.1-03 and 5.1.1-04). In this study, no significant exhalation of radioactivity was observed into the expired air until 48 h postdose, regardless of the sex.

The excretion of radioactivity following oral administration of [azaspirodecenyl-3-^{14}C]BYI 08330 is shown in Table 5.1.1-09.

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III. Conclusions

The fate of radiolabelled BYI 08330 in male and female rats following single and repeated oral administration was characterized as follows:

- **Absorption**: Fast and complete absorption of the radioactive test compound from the gastrointestinal tract for the low dose, high dose, and repeated dose groups of male and female rats
- **Distribution**: Fairly equal distribution of the radioactivity in males and females within the blood and most organs and tissues with preference to the liver and kidney as the main metabolizing organ also responsible for excretion
- Steady decline of radioactivity concentrations in plasma by several orders of magnitude within 48 hours
- Very low concentrations of radioactivity in tissues and organs at the time of sacrifice 48 hours postdose which were <LOD for some organs/tissues
- **Excretion**: Rapid and complete excretion of radioactivity mainly *via* the renal route in all dose groups
- **Metabolism**: Cleavage of the ester group as the major metabolic transformation resulting in BYI 08330-enol as the major metabolite and key intermediate in the metabolic pathway
- Demethylation of the methoxy group in the cyclohexyl ring resulting in BYI 08330-desmethyl-enol as the second prominent metabolite
  This metabolic transformation was significantly higher in male rats than in females
- Further oxidative transformation and conjugation of BYI 08330-enol were of minor importance
- Very similar metabolic profiles for low dose and repeated dose tests. Similar profiles for low dose and high dose tests but with a somewhat higher proportion of BYI 08330-desmethyl-enol in the high dose tests
- Significant difference in metabolic profiles between male and female rats for low dose, high dose and repeated dose groups with male rats exhibiting much higher proportions of BYI 08330-desmethyl-enol than females
- High identification rate of metabolites in all dose groups (87 – 95 % of administered dose)

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Table 5.1.1-05: Excretion and radiolabelled residues in organs and tissues of male and female rats after oral administration of [azaspirodecenyl-3-^{14}C]BYI 08330

Radioactivity in % of dose administered						
Dose [mg/kg] Sex	Test 8 2 male	Test 2 2 female	Test 3 100 male	Test 4 100 female	Test 5 2 (14+1) pretreatm. male	Test 9 2 (14+1) pretreatm. female
Urine Faeces	93.34 5.11	87.92 3.34	89.14 10.51	93.78 2.98	91.48 6.59	94.78 1.78
Total excreted	**98.45**	**91.26**	**99.65**	**96.76**	**98.07**	**96.56**
Skin Sum Organs	0.004 0.019	0.070 0.058	0.015 0.112	0.014 0.021	0.011 0.067	0.024 0.036
Body without GIT	0.023	0.129	0.126	0.035	0.078	0.060
GIT	0.014	0.043	0.012	0.012	0.035	0.024
Total Body	0.038	0.171	0.138	0.047	0.113	0.083
Balance	**98.49**	**91.44**	**99.78**	**96.81**	**98.19**	**96.65**
Radioactivity in % of total recovered radioactivity						
Urine Faeces	94.79 5.18	96.06 3.75	89.34 10.52	96.86 3.10	93.23 6.66	98.06 1.85
Total excreted	**99.97**	**99.81**	**99.86**	**99.96**	**99.89**	**99.91**
Skin Sum Organs	0.004 0.019	0.078 0.064	0.015 0.113	0.014 0.022	0.012 0.069	0.025 0.037
Body without GIT	0.024	0.142	0.127	0.036	0.080	0.062
GIT	0.014	0.047	0.012	0.013	0.036	0.025
Total Body	0.038	0.189	0.139	0.049	0.117	0.086
Norm.-Factor **Absorption rate**	1.015 **94.81**	1.101 **96.20**	1.002 **89.47**	1.034 **96.90**	1.022 **93.31**	1.036 **98.12**

Absorption rate = radioactivity in urine + radioactivity in body without GIT

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Table 5.1.1-06: Time course of radioactivity measured in the plasma of rats after oral administration of [azaspirodecenyl-3-^{14}C]BYI 08330

Equivalent concentration C [µg/g] in plasma (measured values)						
Time (hours post administration)	Test 8 2 mg/kg male	test 2 2 mg/kg female	test 3 100 mg/kg male	test 4 100 mg/kg female	test 5 2 mg/kg (14+1) pretreatm. male	test 9 3 mg/kg (14+1) pretreatm. female
0.08	1.435	2.225	14.436	16.071	1.784	10.764
0.17	2.568	3.836	45.424	52.592	3.935	33.983
0.33	3.396	3.239	101.600	89.249	4.891	65.247
0.67	4.139	2.925	157.374	116.044	4.962	92.793
1.00	4.402	2.394	189.735	115.816	4.383	103.311
1.50	4.004	1.598	204.390	100.464	3.401	102.752
2.00	3.390	1.067	201.130	87.011	2.581	96.907
3.00	2.358	0.659	194.017	58.094	1.661	84.590
4.00	1.558	0.515	168.045	40.126	1.114	69.762
6.00	0.676	0.356	122.464	21.094	0.495	48.018
8.00	0.327	0.280	78.375	16.608	0.246	31.743
24.00	0.005	0.035	0.415	0.204	0.009	0.209
32.00	0.003	0.023	0.189	0.150	0.003	0.114
48.00	0.002	0.011	0.154	0.098	0.002	0.085

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Table 5.1.1-07: Pharmacokinetic parameters obtained by plasma curve analysis using the software program TOPFIT

Parameter	Units	Test 8	Test 2	Test 3	Test 4	Test 5	Test 9
Dose	mg/kg bw	2	2	100	100	2 (14+1) pretreatm.	2 (14+1) pretreatm.
Sex of rats		male	female	male	female	male	female
No. of animals		4	4	4	4	4	3
Mean rat weights (ad administration)	g	202	204	205	202	213	208
Renal elimination	% of dose admin.	93.34	87.92	89.14	93.78	91.48	94.78
Correl. coefficient		0.999	0.996	0.996	0.997	1.000	0.994
C_{max} (model)	µg/g	4.41	4.15	210	117	5.21	2.98
t_{max} (model)	h	0.89	0.09	2.03	0.77	0.45	0.35
C_{max} (experiment)	µg/g	4.40	3.84	204	116	4.96	2.69
t_{max} (experiment)	h	1.00	0.17	1.50	0.66	0.66	0.66
$t_{1/2\ a}$	h	<0.01	<0.01	0.17	0.06	0.10	0.07
$t_{1/2\ e(1)}$	h	0.31	4.79	1.70	0.19	3.62	0.47
$t_{1/2\ e(2)}$	h	20.1	29.7	17.5	27.2	92.7	13.2
$t_{lag\ a}$	h	<0.01	0.08	0.06	0.05	0.03	0.03
$AUC_{(0-\infty)}$	µg/g x h	16.4	10.2	1380	451	14.6	7.64
k_{1e}	1/h	246	258	0.98	3.53	5.61	8.53
CL/f	mL/min/kg	2.03	3.27	1.21	3.70	2.28	4.36
CL_R	mL/min/kg	1.89	2.89	1.08	3.47	2.09	4.13
MRT	h	3.39	9.68	4.90	4.26	5.29	4.38
MRT_{abs}	h	2.55	1.16	2.53	2.99	1.91	1.94
MRT_{disp}	h	0.84	8.52	2.37	1.27	3.38	2.44
Vss	L	0.10	1.67	0.17	0.28	0.46	0.64
weighting function*		g = 1	g = 1/y	g = 1	g = 1/y	g = 1	g = 1
compartments	number	3	3	3	3	3	3
Remarks: $CL_{renal} = CL_{total}$ x % urine excretion x 0.01							

* for some individual data points weighting factors were used for better curve fitting

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Table 5.1.1-08: Distribution of radioactivity in rat tissues/organs at sacrifice, 48 hours after oral administration of [azaspirodecenyl-3-^{14}C]BYI 08330:

Equivalent concentration C [µg/g] (mean values)						
Dose [mg/kg]	Test 8 2	Test 2 2	Test 3 100	Test 4 100	Test 5 2 (14+1) pretreatm.	Test 9 2 (14+1) pretreatm.
Sex	Male	female	male	female	male	female
Erythrocytes	0.0010	0.0013	0.0385	0.0250	0.0007	0.0007
Plasma	0.0011	0.0015	0.0703	0.0267	0.0009	0.0010
Spleen	0.0006	0.0009	0.0626	<LOD	0.0006	0.0006
GIT	0.0024	0.0094	0.0809	0.0999	0.0035	0.0046
Liver	0.0076	0.0035	0.1792	0.0502	0.0094	0.0019
Kidney	0.0009	0.0040	0.1065	0.0609	0.0024	0.0027
Perirenal fat	<LOD	<LOD	<LOD	<LOD	0.0047	<LOD
Adrenal gland	<LOD	<LOD	<LOD	<LOD	0.0062	<LOD
Testis	0.0008	n.a.	0.0622	n.a.	0.0003	n.a.
Ovaries	n.a.	<LOD	n.a.	<LOD	n.a.	<LOD
Uterus	n.a.	<LOD	n.a.	<LOD	n.a.	0.0015
Skel. muscle	<LOD	0.0013	0.0377	<LOD	0.0006	<LOD
Bone femur	<LOD	0.0030	0.0855	0.0534	0.0009	<LOD
Heart	0.0006	0.0010	0.0332	0.0189	0.0006	0.0005
Lung	0.0005	0.0011	0.0327	0.0220	0.0006	0.0007
Brain	<LOD	0.0005	<LOD	<LOD	<LOD	<LOD
Thyroid gland	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Skin	0.0008	0.0060	0.0567	0.0529	0.0008	0.0022
Carcass	<LOD	0.0015	0.1649	0.0257	0.0009	0.0010

Legend:
<LOD = value below detection limit
n.a. = not applicable
The given concentrations are mean values from 4 animals of each test.

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Table 5.1.1-09: Time course of the excretion of radioactivity via urine and faeces of male and female rats after oral administration of [azaspirodecenyl-3-^{14}C]BYI 08330

Cumulative excretion of radioactivity in % of dose administered						
	Test 8	Test 2	Test 3	Test 4	Test 5	Test 9
Dose [mg/kg]	2	2	100	100	2 (14+1) pretreatm.	2 (14+1) pretreatm.
Sex	male	female	male	female	male	female
Urine (time post administration [h])						
4	34.48	36.50	17.95	55.19	40.65	56.35
8	77.48	45.10	50.87	78.95	77.77	57.55
12	89.95	*	*	*	*	*
24	92.96	85.68	88.32	93.03	90.86	93.16
48	93.34	87.92	89.14	93.78	91.48	94.78
Faeces (time post administration [h])						
24	4.89	2.30	9.95	2.79	5.94	1.44
48	5.11	3.34	10.51	2.98	6.59	1.78
Sum excreted	**98.45**	**91.27**	**99.64**	**96.76**	**98.08**	**96.56**

*) no sample collected

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Table 5.1.1-10: Metabolite distribution in the excreta of male and female rats dosed with [azaspirodecenyl-3-^{14}C]BYI 08330

	Percent of administered dose					
	Test 8 2 male	Test 2 2 female	Test 3 100mg/kg male	Test 4 100mg/kg female	Test 5 2 mg/kg rep.dose (14+1) male	Test 9 2 mg/kg rep.dose (14+1) female
Excreted with urines	93.34	87.92	89.14	93.78	91.48	94.78
Excreted with faeces	5.11	3.34	10.51	2.98	6.59	1.78
Excreted with urines and faeces	98.45	91.27	99.64	96.76	98.08	96.56
Extracted from faeces	4.58	2.30	8.71	1.97	5.43	1.33
Compound (report name):						
BYI 08330-						
-enol-GA	0.51	0.16	0.79	0.18	0.43	0.15
-enol-alcohol	1.25	0.42	1.58	0.43	1.20	0.81
-desmethyl-enol	26.93	5.01	37.09	10.06	24.73	5.38
-desmethyl-ketohydroxy	0.31	0.21	0.65	0.06	0.36	0.11
-enol	64.82	80.50	52.92	83.37	66.06	86.66
-ketohydroxy	0.87	1.10	0.49	0.49	0.59	0.80
Total identified	**94.69**	**87.40**	**93.52**	**94.59**	**93.37**	**93.89**
unknown (peak 1)	0.18	---	0.22	---	0.17	---
unknown (peak 3)	0.40	0.02	0.51	---	0.42	0.10
unknown (peak 4)	0.54	---	0.72	---	0.59	---
unknown (peak 5)	0.21	---	0.31	---	0.26	---
unknown (peak 6)	0.39	0.03	0.27	---	0.44	---
unknown (peak 8)	0.02	---	0.23	---	0.03	---
unknown (peak 9)	0.05	0.02	0.06	0.04	0.07	0.03
unknown (peak 10)	0.06	0.02	0.10	---	0.06	0.03
unknown (peak 12)	0.31	0.09	0.34	0.12	0.28	0.14
unknown (peak 13)	0.55	0.13	0.66	0.18	0.49	0.16
unknown (peak 16)	0.13	0.09	0.05	0.06	0.10	0.10
unknown (peak 18)	---	---	---	---	---	0.02
unknown (peak 19)	---	---	0.03	---	---	0.01
Total unidentified	2.85	0.40	3.51	0.41	2.92	0.60
Faeces extracts not analysed	0.07	0.07	0.85	0.73	0.24	0.03
Total characterised = total unidentified and faeces extracts not analysed	2.92	0.47	4.36	1.14	3.16	0.62
Solids after faeces extraction	0.23	0.11	0.39	0.09	0.27	0.09
Urines not analysed (24-48 h)	0.38	2.24	0.82	0.75	0.62	1.62
Faeces not analysed (24-48 h)	0.22	1.05	0.56	0.19	0.66	0.34
Total excreta samples not analysed	0.84	3.39	1.77	1.03	1.54	2.05
Total	**98.45**	**91.26**	**99.65**	**96.76**	**98.07**	**96.56**

--- = not detected

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Figure 5.1.1-01 Proposed metabolic pathway of BYI 08330 in rats

BYI08830

BYI08330-enol-alcohol

BYI08330-enol

BYI08330-enol-GA

BYI08330-ketohydroxy

BYI08330-desmethyl-enol

BYI08330-desmethyl-ketohydroxy

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Report:	**KIIA 5.1.1/03, 2006**
Title:	Physiology based pharmacokinetic simulation of BYI 08330 in male rats
Report No & Document No	BTS-WSM0602 M-274844-01-2
Guidelines:	Not applicable
GLP	No (simulation)
Testing Facility and Dates	, Germany Experimental work: Simulation, no experimental study

Executive Summary

A physiology based pharmacokinetic (PBPK) analysis of ADME data of BYI 08330 for male rats (Report No. MEF-048/04) and results of the Quantitative Whole Body Autoradiography study (Report No. MEF-06/15) revealed indications of the presence of saturable processes that influence the pharmacokinetics of the compound. These indications are a change in shape of the plasma concentration curve with increasing dose and extraordinarily high concentrations in liver and kidney compared to other tissues. Both organs are known to express various active transport proteins with a broad variety of substrates.

It could be shown that the experimentally determined ADME behaviour of BYI 08330 in male rats can be well described by PBPK simulations assuming that the compound enters the systemic circulation as the metabolite BYI 08330-enol that is than further metabolized to BYI 08330-desmethyl-enol. The time course of observed plasma concentrations, their non-linearity in dose, organ concentrations and the metabolization and excretion could be described with very good agreement by simulations based on a common parameterization of the PBPK model.

Based on an analysis of experimental and simulation results the following conclusions about the ADME behaviour of BYI 08330 can be drawn:

- Strongly increased concentrations in liver and kidney compared to plasma and other tissues, as observed by QWBA, indicate the presence of active transport processes for the uptake of BYI 08330 metabolites into these tissues. Inclusion of such processes in the simulation led to a very good agreement between calculated and observed concentrations in both organs.
- The renal excretion rate of BYI 08330-enol can only be explained by active tubular secretion into urine, because the physicochemical properties yield a glomerular filtration rate that is much smaller than the observed rate of excretion.
- With a high probability a certain saturation of the renal transport processes is responsible for the change in shape of plasma concentration curves observed at 100 mg/kg, although the available experimental data do not allow to decide which of both processes, uptake or secretion, is the more relevant one.
- The extent of metabolization of BYI 08330-enol to BYI 08330-desmethyl-enol, determined by the desmethyl-enol fraction in excreta, is in agreement with the rather low metabolic rate measured in-vitro as could be shown by simulations using the in-vitro metabolization rate. This is due to the disproportionate hepatic concentration.

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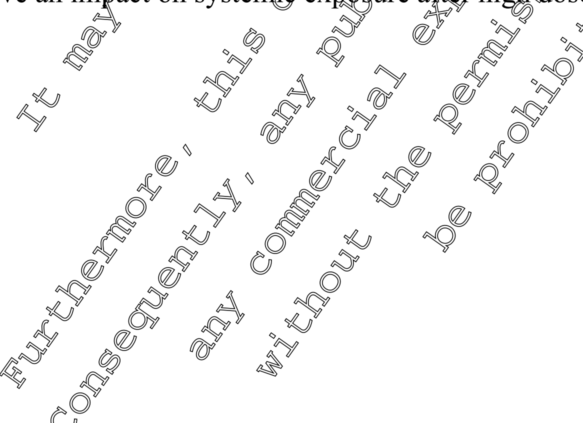
Extrapolation of pharmacokinetics was carried out for doses up to 1000 mg/kg, which were significantly higher than those investigated experimentally in the ADME study (maximum 100 mg/kg) and which represent dose levels relevant in toxicological studies.
The most important results of the extrapolation were:

- The saturation of the active transport processes leads to a strong change of the shape of the plasma concentration curve at high doses resulting in a plateau-like concentration time curve due to a decreased efficiency of excretion.
- The non-linearity of plasma concentration with dose levels leads to a disproportionate increase of systemic exposure with increasing dose. While the dose normalized maximum concentrations show only a fair increase with dose, this increase is rather strong for the area under the concentration curve. The dose normalized area under concentration curve (AUC_{norm}) is about five times higher at 1000 mg/kg than at 2 mg/kg.
- The peak/trough ratio ($C_{max}/C_{(24h)}$) changes even more pronounced with dose. It decreases roughly by a factor of 500 reaching values as low as 5 – 6 at 1000 mg/kg, thus being indicative of a rise of systemic concentrations upon repeated daily administration.

In order to assess the impact of this non-linear behaviour on toxicological studies with repeated administrations the dose dependence of plasma concentrations during a four weeks period with daily administrations was simulated. In this case, a rise of plasma concentrations was found for daily doses above 500 mg/kg. At 1000 mg/kg, the mean daily concentrations increased with the time by about a factor of two until a steady state was reached after about 15 days. This caused an even higher non linearity of the AUC, with a 7-fold increase of AUC_{norm} with doses increasing from 2 mg/kg to 1000 mg/kg, compared to 5-fold for a single administration.
An assessment of the dependence of the disproportional increase in systemic exposure on the peak/trough ratio showed that a distinct change in behaviour occurs between peak/trough ratios of 5 and 3. Below that range the moderate temporal increase of systemic exposure described above changes into a strong one with concentrations increasing continuously at least over the whole four weeks investigated here. This leads to systemic exposures (AUC) at high doses which are about 20 times higher than those extrapolated linearly from low dose values.
The disproportionate increase in plasma concentration after repeated administration of BYI 08330 might have an impact on systemic exposure after high doses in toxicological studies.



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I. Material and Methods

A. Material

No test material was used since this report includes a simulation and not an experimental study.

B. Study Design

Pharmacokinetic simulation

Physiology based pharmacokinetic simulations were carried out with the commercially available software PK-Sim (Bayer Technology Services GmbH). PK-Sim is based on a generic whole body PBPK-model which describes uptake and distribution of organic substances after oral or intravenous administration. Results of PBPK-simulations with PK-Sim consist of concentration time curves for all organs and compartments included in its PBPK-model (Venous Blood, Arterial Blood, Bone, Brain, Fat, Heart, Kidney, Large Intestine, Liver, Lung, Muscle, Pancreas, Portal, Skin, Small Intestine, Spleen, Stomach, Gonads). The concentrations are given as total tissue concentrations or as total or unbound concentrations in interstitial or cellular space for the organs. Blood concentrations are available as unbound or total plasma or red blood-cell concentrations. In order to account for a specific fate of the simulated substance PK-Sim allows to describe metabolization in all included organs as well as an active transport across all permeation barriers considered in the simulation model.

For the physiological parameters of the model (blood flow rates, volumes of organs and their sub-compartments) default values are provided by PK-Sim itself for the species mouse, rat, dog and human. These physiological parameters can be set to customized values for the simulation of special scenarios as e.g. disease states or particular animal models. Organ/plasma partition coefficients and passive permeabilities are estimated by PK-Sim from physicochemical properties of the compound to be simulated. All other substance specific model parameters (e.g. clearance, metabolic rates, rates of active transport) have to be entered directly as input values. Their values must either be known from dedicated experiments or can be estimated by manually adjusting the simulation for the best fit to experimental pharmacokinetic data.

A starting parameterization of the generic PBPK-model of PK-Sim was achieved as follows:

<u>Physiological parameters:</u>

All values parameters depending solely on physiology and anatomy of the simulated individual were kept as provided by the built-in database of PK-Sim.

<u>Compound specific parameters:</u>

Parameters depending on the substance to be simulated were in first instance defined using the PK-Sim functionality to calculate the parameters of passive uptake and distribution processes from physicochemical data. Because no parent compound was observed in the blood plasma of rats after oral administration of BYI 08330, the first parameterization was made according to the properties of the major metabolite BYI 08330-enol. The predominant excretion route for BYI 08330-enol is renal excretion. Thus the total clearance was in first instance ascribed to renal clearance.

The starting parameterization was made by setting values of the physicochemical properties of BYI 08330-enol and the clearance to the values given in the table below.

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PK-Sim input parameter	Value	Comment
Lipophilicity	1.76	LogD(pH2) determined by RP-HPLC
Plasma binding	-5.3	Logarithmic dissociation constant for human serum albumin calculated from chemical structure using in house QSAR method.
Molecular Weight [g/mol]	301 g/mol	
Solubility [mg/l]	2200 mg/l	Water solubility calculated for pH6.5 from experimental value of 159 mg/l at pH5
Hepatic Clearance [ml/min/kg]	0	
Renal Clearance [ml/min/kg]	1.5 ml/min/kg	Obtained by fitting simulation to plasma concentrations of study with dose = 2 mg/kg

The following experimental data were used within the study for correlation to simulation results.

Reference No. *)	Report No. and Date	Author	Title	Data used
2006b	MEF-048/04 2006		[Azaspirodecenyl-3-^{14}C]BYI 08330: Adsorption, Distribution, Excretion and Metabolism in the Rat	- Plasma concentrations (total radioactivity) at doses of 2 mg/kg and 100 mg/kg. - Metabolite spectrum in excreta at 2 mg/kg and 100 mg/kg
2006a	MEF-06/15 2006		[Azaspirodecenyl-3-^{14}C]BYI 08330: Distribution of the Total Radioactivity in Male and Female Rats Determined by Quantitative Whole Body Autoradiography (QWBA) Including Determination of the Total Radioactivity in Excreta and Exhaled $^{14}CO_2$	- Tissue-concentrations (total radioactivity) in peripheral organs
2006	SA 05319 2006		[Azaspirodecenyl-3-14C]-BYI 08330: Comparison of the in Vitro metabolism in Liverbeads™ from rat, mouse and human.	- Fraction of desmethyl-enol detected after incubation with 50 µM BYI 08330 for 4 h.

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The following modelling calculations were performed in the study:

- concentration time curves for total radioactivity in plasma after a single oral dose of 2 mg/kg bw in comparison to ADME study
  simulation based on passive distribution alone
- concentration time curves for total radioactivity in organs and tissues after a single oral dose of 3 mg/kg bw in comparison to quantitative whole body autoradiography study
    - simulation based on passive distribution alone
    - simulation based on refined model including active uptake into liver and kidney parameters for active uptake optimized for best fit of simulated data to experimental results
- concentration time curves for total radioactivity in plasma after a single oral dose of 2 mg/kg bw in comparison to ADME study
  simulation based on refined model including active uptake into liver and kidney using optimized parameters derived from modelling of concentration time curves in organs and tissues
- concentration time curves for total radioactivity in plasma after a single oral dose of 2 mg/kg bw and 100 mg/kg bw in comparison to ADME study
    - simulation based on refined model including active uptake into liver and kidney plus <u>saturation of renal uptake</u> at high dose level
    - simulation based on refined model including active uptake into liver and kidney plus <u>saturation of renal excretion</u> at high dose level
- extrapolation of concentration time curves for total radioactivity in plasma after a single oral dose for high dose levels up to 1000 mg/kg bw not studied in the experiment
    - simulation based on refined model including active uptake into liver and kidney plus <u>saturation of renal uptake</u> at high dose level
    - simulation based on refined model including active uptake into liver and kidney plus <u>saturation of renal excretion</u> at high dose level
    - simulation including in addition <u>saturation of hepatic uptake</u>
- dependence of AUC (area under the curve), C_{max} (maximal plasma concentration) and peak/trough ratio (C_{max}/C_{24h}) from dose level for single doses (dose range 2 – 1000 mg/kg bw).
- dependence of dose normalized AUCnorm (dose normalized area under the curve) and Cmax (dose normalized maximal plasma concentration) from dose level repeated dosing during a 28 days period (dose range 2 – 1000 mg/kg bw).

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II. Results and Discussion

It could be shown that the ADME behaviour of BYI 08330 in male rats can be well described by physiology based simulations as far as it is investigated experimentally. It was possible to find a single parameterization of the PBPK model in such a way that observed plasma concentrations, their non-linearity in dose, organ concentrations and the metabolization and excretion could be commonly described by the simulation with excellent agreement to experimental data.

Strongly increased concentrations in liver and kidney compared to plasma and other tissues, as observed by quantitative whole body autoradiography (Report No. MEF-06/15), indicated the presence of active transport processes for the uptake of BYI 08330 metabolites into these tissues.

Moreover the renal excretion rate of BYI 08330-enol could only be explained by active tubular secretion into urine, because the physicochemical properties yielded a glomerular filtration rate that was much smaller than the observed rate of excretion. It could be shown with a high probability that the saturation of the renal transport processes is responsible for the experimentally observed non-linearity of plasma concentrations between the doses 2 mg/kg and 100 mg/kg (Report No. MEF-048/04). While the available experimental data did not allow to determine, which of the two processes, uptake or excretion, is the more relevant one, it is reasonable to assume that in reality there is a saturation of both processes simultaneously, since the unbound concentrations comparable to typical binding constants (in the µmol range) for transport proteins are reached or exceeded in the plasma as well as the kidney cells.

The PBPK-simulations allowed an extrapolation to doses higher than those investigated experimentally in ADME studies. Calculations were carried out for doses up to 1000 mg/kg, covering the range that is studied in toxicological experiments. It was found that the saturation of the active transport processes leads to a strong change of the shape of the plasma concentration curve at the highest doses. This has in turn a significant impact on pharmacokinetic parameters, describing the systemic exposure to a compound. While the dose-normalized maximum concentrations only show a fair increase with the dose, this increase is rather strong for the area under the concentration curve.

An even more pronounced change was found for the peak/trough ratio ($C_{max}/C_{(24h)}$). This parameter decreases roughly by a factor of 500 reaching values as low as 5 – 6 at 1000 mg/kg. Such low peak/trough ratios are indicative of a potential continuous rise of systemic concentrations upon repeated daily administration. In fact such rise of body burden was found for doses above 500 mg/kg in simulations of daily administration over a period of four weeks. At 1000 mg/kg, the mean daily concentrations increased about twofold with the time until a steady state was reached after about 15 days. This led to an even higher non linearity of the AUC, with a 7-fold increase of AUC_{norm} between 2 mg/kg and 1000 mg/kg, compared to 5-fold at single administration.

A sensitivity analysis revealed that under certain circumstances also peak/trough ratios being distinctly below 5 will be obtained. This is particularly the case if either the fraction of the dose absorbed from the gut is higher or the metabolic rate for the transition enol to the desmethyl-enol is lower at high doses than regarded in the present simulations. The actual values of the respective properties might generally differ from those of the simulation because they rely on parameter values estimated with some uncertainty.

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An assessment of the dependence of the disproportional increase in systemic exposure on the peak/trough ratio showed that a distinct change in the behaviour occurs between ratios of 5 and 3. Below that range the moderate temporal increase of systemic exposure described above changes into a strong one with concentrations increasing continuously at least over the whole four weeks investigated here. This leads to predicted systemic exposures (AUC) at very high doses which are about 20 times higher than those extrapolated linearly from low dose values.

III. Conclusions

An analysis of ADME data of BYI 08330 for male rats (Report No. MEF-048/04) and results of the Quantitative Whole Body Autoradiography study (Report No. MEF-06/15) revealed indications of the presence of saturable processes that influence the pharmacokinetics of the compound. These indications are a change in shape of the plasma concentration curve with increasing dose and extraordinarily high concentrations in liver and kidney compared to other tissues. Both organs are known to express various active transport proteins with a broad variety of substrates.

It could be shown that the experimentally determined ADME behaviour of BYI 08330 in male rats can be well described by PBPK simulations assuming that the compound enters the systemic circulation as the metabolite BYI 08330-enol that is than further metabolized to the BYI 08330-desmethyl-enol. The observed plasma concentrations, their non-linearity in dose, organ concentrations and the metabolization and excretion can be described with very good agreement by simulations based on a common parameterization of the PBPK model.

Based on an analysis of experimental and simulation results the following conclusions about the ADME behaviour of BYI 08330 can be drawn:

- Strongly increased concentrations in liver and kidney compared to plasma and other tissues, as observed by QWBA, indicate the presence of active transport processes for the uptake of BYI 08330 metabolites into these tissues. Inclusion of such processes in the simulation led to a very good agreement between calculated and observed concentrations in both organs.
- The renal excretion rate of BYI 08330-enol can only be explained by active tubular secretion into urine, because the physicochemical properties yield a glomerular filtration rate that is much smaller than the observed rate of excretion.
- With a high probability, a certain saturation of the renal transport processes is responsible for the change in shape of plasma concentration curves observed to occur at 100 mg/kg, although the available experimental data do not allow to decide which of both processes, uptake or secretion, is the more relevant one.
- The extent of metabolization of BYI 08330-enol to BYI 08330-desmethyl-enol, determined by the desmethyl-enol fraction in excreta, is in agreement with the rather low metabolic rate measured in-vitro as could be shown by simulations using the in-vitro metabolization rate. This is due to the disproportionate hepatic concentration.

Extrapolation of pharmacokinetics were carried out for doses up to 1000 mg/kg, which were significantly higher than those investigated experimentally in the ADME study (maximum 100 mg/kg) and which represent dose levels relevant in toxicological studies.. The most important results of the extrapolation are:

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- The saturation of the active transport processes leads to a strong change of the shape of the plasma concentration curve at high doses resulting in a plateau-like concentration time curve due to a decreased efficiency of excretion.
- The non-linearity of plasma concentration with dose levels leads to a disproportionate increase of systemic exposure with increasing dose. While the dose normalized maximum concentrations show only a fair increase with dose, this increase is rather strong for the area under the concentration curve. The dose normalized area under concentration curve (AUCnorm) is about five times higher at 1000 mg/kg than at 2 mg/kg.
- The peak/trough ratio (Cmax/C(24h)) changes even more pronounced with dose. It decreases roughly by a factor of 500 reaching values as low as 5 – 6 at 1000 mg/kg, thus being indicative of a rise of systemic concentrations upon repeated daily administration.

In order to assess the impact of this non-linear behaviour on toxicological studies with repeated administrations the dose dependence of plasma concentrations during a four weeks period with daily administrations was simulated. In this case a rise of plasma concentrations was found for daily doses above 500 mg/kg. At 1000 mg/kg the mean daily concentrations increased with time by about a factor of two until a steady state was reached after about 15 days. This caused an even higher non linearity of the AUC, with a 7-fold increase of AUCnorm with doses increasing from 2 mg/kg to 1000 mg/kg, compared to 5-fold for a single administration.

The disproportionate increase in plasma concentration after repeated administration of BYI 08330 might have an impact on systemic exposure after high doses in toxicological studies.

Report:	**KIIA 5.1.1/04, 2006**
Title:	[Azaspirodecenyl-3-^{14}C]BYI 08330: Depletion of Residues and Metabolites in Plasma, Urine, Liver, Kidney and Testis of the Male Rat
Report No & Document No	MEF 06/328 M-275731-01-2
Guidelines:	US EPA Guideline No.: OPPTS 870.7485 Metabolism and Pharmacokinetics, EPA Ref.: 712-C-98-244, August 1998 EU Council Directive 91/414/EEC amended by the Commission Directive 94/79/EC, adopted December 21, 1994 OECD Guideline for Testing Chemicals, 417: Toxicokinetics adopted April 04, 1984 Japanese MAFF New Test Guidelines for Supporting Registration of Chemical Pesticides, 12 Nousan 8147, adopted November 24, 2000, amended June 26, 2001
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

, Germany

Experimental work: 2006-05-03 – 2006-06-09

Executive Summary

Three groups of 4 male rats were administered by oral gavage with a single dose of [azaspirodecenyl-3-^{14}C] BYI 08330 in aqueous Tragacanth® at a target dose level of 2 mg/kg body weight and three other groups at a dose level of 1000 mg/kg body weight. One group of animals from each dose level was sacrificed at 1 h, 8 h, and 24 h after dosage, respectively.
The total radioactivity that included parent compound and metabolites was determined in the excreted urine and faeces samples over the testing time as well as in plasma, testis, liver and kidney at sacrifice. Between 100.3 % and 121.8 % (tests 1 – 3) as well as 94.8 % and 98.9 % (tests 4 – 6) of the administered dose were recovered from measurements of the total radioactivity in urine and faeces samples, and in organs and tissues at sacrifice.
Investigations on metabolites were performed with selected urine and plasma samples, and with extracts from testis, liver and kidney. The identification rate was high and accounted for more than 89 % of the TRR for all samples. For most samples, the identification rate was more than 95 %.

In low dose tests, the administered radioactivity was rapidly absorbed, widely distributed into the organs and tissues and rapidly eliminated from the body. Excretion was mainly renal and nearly completed 24 h after dosage. Absorption and excretion were slower and lower in high dose tests. Only 27 % of the dose was renally excreted 24 h after dosage. The balance, time course of excretion and residue levels are summarized in Table 5.1.1-11 and Table 5.1.1-12.

In low dose tests, the residues in plasma and organs declined rapidly from the maximum value after dosage giving low residues in plasma after 24 h post administration. For all time points, the residues in liver and kidney were distinctively higher than in plasma. This finding may indicate that metabolites are transferred by active transport mechanisms from plasma to the excretory organs. The residues in testis, carcass and skin were distinctively lower than in plasma.
The metabolic profiles in urine were in good accordance with the results of the ADME study (Report No. MEF-048/04). The ratio of the two main metabolites BYI 08330-enol and BYI 08330-desmethyl-enol in urine was about 2: 1.
BYI 08330-desmethyl-enol was found at higher percentages in urine than in plasma and organs. This is probably caused by the more rapid excretion of this more polar metabolite compared to BYI 08330-enol. The highest percentages of BYI 08330-desmethyl-enol in the body were detected in liver, where the compound is formed by metabolic transformation of BYI 08330-enol. The percentages in plasma, kidney and testis were comparable and significantly lower than in liver. The metabolic profiles are summarized in Table 5.1.1-13 (urine) and Table 5.1.1-14 (plasma and organs).

In high dose tests, the depletion of residues was distinctively slower and the residues in plasma were slightly higher than in liver and kidney. This may be caused by the saturation of the active transport

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mechanisms resulting in a more uniform distribution of the compound in the body. The residues declined hardly from 1 h to 8 h after dosage and slightly more until 24 h. The residues in testis, carcass and skin were lower than in plasma.
The metabolism was similar to the metabolism in low dose tests, with the exception that BYI 08330-desmethyl-enol was found at higher percentages in high dose tests.
As in low dose tests, BYI 08330-desmethyl-enol was found at higher percentages in urine than in plasma and organs. The highest percentages of BYI 08330-desmethyl-enol were detected in liver as well as in kidney. The percentages in plasma, kidney and testis were lower and comparable. The metabolic profiles are summarized in Table 5.1.1-13 (urine) and Table 5.1.1-15 (plasma and organs).

In all tests, the first and most important metabolic reaction was the cleavage of the ester bond of the side chain yielding the BYI 08330-enol. The demethylation of the cyclohexyl-O-methyl group to the respective alcohol (BYI 08330-desmethyl-enol) was a further important metabolic reaction as well as the hydroxylation in the azaspiro ring of BYI 08330-enol resulting in BYI 08330-ketohydroxy, which was mainly detected in liver and kidney. Other metabolic reactions like conjugation of the BYI 08330-enol with glucuronic acid, oxidation of one of the methyl groups of the phenyl ring forming the BYI 08330-enol-alcohol, and demethylation of BYI 08330-ketohydroxy to BYI 08330-desmethyl-ketohydroxy were of minor importance. The proposed biotransformation pathway of [azaspirodecenyl-^{14}C]BYI 08330 is given in Figure 5.1.1-02.

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Based on all results, it can be concluded that due to the saturation of active transport mechanisms in the excretory organs after administration of high doses, the depletion of residues and the excretion via urine and faeces is slow resulting in a potential for accumulation of BYI 08330 related residues in the body following repeated high doses.

I. Material and Methods

A. Material

1. Test Material

IUPAC Name:	**cis-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate**
Code name:	**BYI 08330**
Common name:	**Spirotetramat (proposed ISO)**
Empirical formula:	**$C_{21} H_{27} N O_5$**
Molar mass:	**373.45 g/mol**
Water solubility:	**pH 4 = 33.5 mg/L, pH 7 = 29.9 mg/L pH 9 = 19.1 mg/L (unstable) all at 20°C**
n-Octanol/water partition coefficient:	**pH 4 = 2.51, pH 7 = 2.51, pH 9 = 2.50**
Labelling:	**azaspirodecenyl-3-^{14}C**
Specific radioactivity of the original radiolabelled batches:	**3.67 MBq/mg (99.1 µCi/mg)**
Specific radioactivity used for administration:	**Low dose tests (test 1 - 3): 3.67 MBq/mg (99.1 µCi/mg) High dose tests (test 4 -6): 0.0073 MBq/mg (0.20 µCi/mg)**
Radiochemical purity:	**> 98 % (certified, HPLC and TLC with radiodetection)**
Dose level:	**2 mg/kg and 1000 mg/kg body weight**
Vehicle:	**0.5 % aqueous tragacanth suspension**
Stability of the test material:	**The stability of [azaspirodecenyl-3-^{14}C]BYI 08330 was demonstrated by radio HPLC analysis of the administration suspensions of each test immediately after dosing**

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2. Test Animals

Species:	**Rat (*Rattus norvegicus domesticus*)**
Strain:	**Wistar Hsd/Cpb: WU**
Breeding facility:	**, Germany**
Sex and numbers involved:	**Males: 24**
Age:	**8 weeks at the time of delivery**
Body weight:	**Males: 194 – 205 g at the time of administration 175 – 204 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 22 - 27 °C, relative humidity 43 – 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 , 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

Justification of the dose levels

The low dose level was selected at 2 mg/kg bw to match the dose of the low dose tests in the ADME study with BYI 08330 (Report No. MEF-048/04). The high dose was selected taking into account the findings from a rat mechanistic study (Report No. SA 04181), where degenerative effects on round / elongating spermatids in the testis of the male rat were found after >10 repeated doses of 1000 mg/kg bw/day. Therefore, 1000 mg/kg bw was selected as the high dose in this study.

Dosing

Each of the rats in the low dose groups (4 rats per group) of test 1 - 3 orally received 2 mg/kg bw of azaspirodecenyl-3-^{14}C BYI 08330 suspended in 0.5 % aqueous tragacanth after ca. 16 h of fasting. The dosing suspension (0.2 mg/mL) was prepared one day before dosing and stored at 4°C. Each of the rats in the high dose groups (4 rats per group) of test 4 - 6 orally received 1000 mg/kg bw of azaspirodecenyl-3-^{14}C BYI 08330 suspended in 0.5 % aqueous tragacanth after ca. 16 h of fasting. The dosing suspension (100 mg/mL) was prepared three days before dosing and stored at 4°C.

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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 administered radioactivity amounts by the specific radioactivity. The actual mean administered dose of [azaspirodecenyl-3-^{14}C]BYI 08330 was 1.83 mg/kg bw for the low dose groups and 934 mg/kg bw for the high dose groups. The stability of the test compound in the suspensions was assured by radio HPLC analysis of aliquots after administration.

Collection of excreta

After administration of the radiolabelled test item, the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine and faeces. For test 1 and test 4 (sacrifice 1 h after dosing), urine was collected separately for each animal in a cryogenic trap cooled with dry ice for the interval of 1 h, faeces were combined with the gastro-intestinal tract at the time of sacrifice. For test 2 and test 5 (sacrifice 8 h after dosing), urine was collected separately for each animal in a cryogenic trap cooled with dry ice in intervals of 4 h and 8 h, faeces were combined with the gastro-intestinal tract at the time of sacrifice. For test 3 and test 6 (sacrifice 24 h after dosing), Urine was collected separately for each animal in a cryogenic trap cooled with dry ice in intervals of 4 h, 8 h, and 24 h, faeces were collected for the interval 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.
The faeces samples were lyophilised (freeze-dried), weighed, and homogenised. The radioactivity was determined by combustion/LSC.

Sacrifice and organ/tissue sampling

At the respective test end (1 h, 8 h or 24 h after administration), the animals were anaesthetised using Pentobarbital-Na. They were sacrificed by transection of the cervical blood vessels. The collected blood was separated into plasma and erythrocytes by centrifugation. The following organs/tissues were collected: erythrocytes, plasma, gastro-intestinal tract (GIT), liver, kidneys, testis, skin, and carcass.
Erythrocytes, GIT, carcass, and skin prepared at the end the experiments were weighed immediately after the dissection and again following lyophilisation. Finally, they were homogenised and the radioactivity determined by combustion/LSC.
Plasma samples were combined for all animals of each test. Liver, kidneys, and testis were weighed and pooled for all animals of each test for the extraction of radioactive residues.

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Radioactivity measurement

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC) of 1 – 5 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 equal to 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.

Metabolite analysis

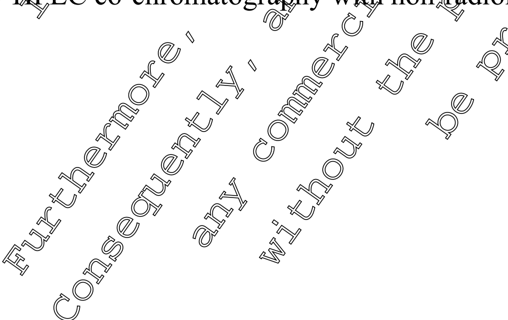
Urine samples were combined for all animals of a test group and analysed by HPLC without prior sample preparation. Combined plasma samples (e.g. 2 mL) were mixed with 5 % formic acid (0.5 mL) and centrifuged. The supernatants were analysed by radio-HPLC.

The livers, kidneys, and testis of all animals of a test group were combined and cut in small pieces. Then, the composite samples were mixed with acetonitrile/water and defoamer and extracted by maceration with a Polytron or Ultraturrax homogeniser. Extracts and solids were separated by centrifugation or filtration and the solids were further extracted with acetonitrile/water (7/3, v/v) as described above. The volumes of the extracts were measured and aliquots radioassayed by LSC.

The post extraction solids were dried at room temperature and aliquots were solubilzed with a tissue solubilizer and radioassayed by LSC. All extracts were combined, filtered, mixed with 0.1 mL emulsifier and evaporated to the aqueous remainder which was analysed by radio-HPLC for metabolic profiling.

The metabolites in the samples were quantified by HPLC using a reversed phase column (C18) and the eluting solvents water/formic acid = 99: 1 (v/v) and acetonitrile/water/formic acid = 97: 2: 1 (v/v/v) in the gradient mode. Detection was performed by a UV (254 nm) and a radioisotope detector with a glass scintillator.

Metabolites in urine were identified by comparison of the elution pattern of the samples from this study with the urines of male rats (low dose test no. 8 dosed with 2 mg/kg bw) from the ADME study (Report No. MEF-048/04). For plasma and extracts of organs, metabolites were assigned by comparison of profiles with those of urine. Furthermore, the most prominent metabolites BYI 08330-enol, BYI 08330-desmethyl-enol and BYI 08330-ketohydroxy were identified representatively in urine and liver extract by HPLC co-chromatography with non-radiolabelled reference items.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II. Results and Discussion

A. Distribution and residues in organs and tissues

In <u>low dose</u> test group 1, sacrificed 1 h after dosing, 33.1 % of the dose were detected in the organs and tissues, 32.1 % in the GIT (plus faeces for test 1 and 2) and 12.5 % in urine. About 66 % of the administered dose were already excreted via urine 8 h after administration in test group 2 resulting in accordingly distinctively lower residues in organs and tissues of only about 12 % of the dose and about 9 % in the GIT. The fast excretion and decline of residues in the body continued, so that 24h after administration only 0.1 % of the dose were found in organs and tissues and 0.2 % in the GIT, but 112 % in urine.

The highest residues in the organs were detected in liver and kidney, which are the primary organs for metabolism and excretion. From 1 h to 24 h, the values in liver and kidney decreased from 23 % (liver) and 5 % (kidney) of the dose to <0.1 % of the dose. The corresponding TRR values of liver and kidney were nearly identical 1 h after administration (11.7 and 11.2 mg/kg) with a rapid decline to 0.02 and 0.01 mg/kg within 24 h.

The radioactivity in plasma was 3.5 % of the administered dose 1h after dosage showing the same fast decline as the other organs to <0.1 % of the dose 24 h after dosage. The TRR values of plasma were distinctively lower than in liver and kidney, but showed a similar fast decline from 4.5 mg/kg after 1 h down to <0.01 mg/kg after 24 h. The TRR values in testis were much lower than in plasma for all time points (0.7 mg/kg at 1 h declining to <0.01 mg/kg at 24 h).

In <u>high dose</u> test group 4, sacrificed 1 h after dosing, only 2.4 % of the dose was detected in the organs and tissues, 85.9 % in the GIT (plus faeces for test 4 and 5) and 1.6 % in urine. Only 16 % of the administered dose was excreted via urine 8 h after administration in test group 5. The residues declined slowly from 1 h to 8 h from 2.4 % to 1.8 % of the dose in organs and tissues and from 86 % to 73 % of the dose in the GIT. From 8h to 24h, the decline of radioactive residues was slightly higher resulting in 0.7 % of the dose in organs and tissues, 47 % in the GIT. In urine, 27 % were found at 24h post dosage.

In contrast to low dose tests, the highest TRR values 1 h and 8 h after dosage were not detected in liver or kidney, but in plasma. The TRR values in plasma were about 10 - 20 % higher than in liver and kidney and amounted to 353 mg/kg in plasma after 1h and to 271 mg/kg in plasma after 8h (liver: 315 mg/kg and 246 mg/kg, and kidney: 308 mg/kg and 222 mg/kg). The decline in all organs was slow from 1 h to 8 h and slightly faster from 8 h to 24 h with 102 mg/kg for plasma, 109 mg/kg for liver and 85 mg/kg for kidney. The TRR values in testis were much lower than in plasma for all time points (66 mg/kg at 1 h, 77 mg/kg at 8 h and 28 mg/kg at 24 h).

The total radioactive residues detected in plasma, organs and tissues at sacrifice (expressed as equivalent concentration C, as dose normalised concentration CN and as percentage of administered dose) are given in Table 5.1.1-12.

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B. Metabolism

In the low dose tests, parent compound was not detected in any sample or tissue. BYI 08330-enol was the main metabolite in all samples. BYI 08330-desmethyl-enol was the second prominent metabolite in urine, plasma and testis, whereas in liver and kidney BYI 08330-ketohydroxy was found as the second prominent metabolite. BYI 08330-ketohydroxy was only present at trace levels in plasma, testis and urine. BYI 08330-enol-GA, -enol-alcohol and –desmethyl-ketohydroxy were detected at low levels. Therefore, they are of minor importance. Residues in all tissues and plasma 24 h after dosage were too low for quantification of metabolites. Identifications rates were high in the range of 89 – 100 %.
The metabolic profiles for urine were in good agreement with the results of the low dose tests in the ADME study.
A summary of the metabolism in urine is presented in Table 5.1.1-13; the metabolism in plasma and organs is summarized in Table 5.1.1-14.

Similar results were obtained for the high dose tests. Parent compound was not detected in any sample or tissue. BYI 08330-enol was the main metabolite in all samples. BYI 08330-desmethyl-enol was the second prominent metabolite in urine, plasma and organs. Significant proportions of BYI 08330-ketohydroxy were present in liver and kidney, only. BYI 08330-enol-GA, BYI 08330-enol-alcohol and BYI 08330-desmethyl-ketohydroxy were detected at low levels and were of minor importance. Identifications rates were high in the range of 95 – 100 %. A summary of the metabolism in urine is presented in Table 5.1.1-13; the metabolism in plasma and organs for the high dose is summarized in Table 5.1.1-15.

C. Excretion

The urinary excretion was fast in the low dose test groups 1 – 3 and almost completed within the first day after administration. About 92 % of the recovered radioactivity (equivalent to 112 % of the administered dose) was excreted via urine. About 8 % of the recovered radioactivity (equivalent to 10 % of the administered dose) were detected in faeces of test 3, 24 h after dosage.
In high dose test groups 4 – 6, excretion was distinctively slower than in low dose ones. Only 27 % of the administered dose were excreted via urine 24 h after dosage and 18 % via faeces, respectively. The major part of the radioactivity was recovered in the gastro-intestinal tract of animals. Detailed data for excretion and residues in organs and tissues are presented in Table 5.1.1-11.

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III. Conclusions

The first and most important metabolic reaction was the cleavage of the ester bond of the side chain yielding the BYI 08330-enol. The demethylation of the cyclohexyl-O-methyl group to the respective alcohol (BYI 08330-desmethyl-enol) was a further important metabolic reaction as well as the hydroxylation in the azaspiro ring of BYI 08330-enol resulting in BYI 08330-ketohydroxy. Other metabolic reactions like conjugation of the BYI 08330-enol with glucuronic acid and oxidation of one of the methyl groups of the phenyl ring forming the BYI 08330-enol-alcohol were of minor importance. The results of the <u>low dose tests</u> were in good accordance to those of the low tests from the ADME study (Report No. MEF-048/04):

- Absorption, distribution in the body and excretion of the dose administered were fast. Excretion was mainly renal and nearly completed 24 h after dosage.
- The residues in plasma and organs declined rapidly from the maximum value 1 h after dosage giving low residues in plasma 24 h post administration.
- For all time points, the residues in liver and kidney were distinctively higher than in plasma. This finding may indicate that metabolites are transferred by active transport mechanisms from plasma to the excretory organs.
- The residues in testis, carcass and skin were distinctively lower than in plasma proving the fast excretion of the residues and the non-accumulation of the compound.
- The metabolic profiles in urine were similar to the results of the ADME study.
- The ratio of the two main metabolites BYI 08330-enol and BYI 08330-desmethyl-enol in urine was about 2: 1.
- BYI 08330-desmethyl-enol was found at lower proportions in plasma and organs with BYI 08330-enol / BYI 08330-desmethyl-enol ratios of 10: 1 up to 40: 1. This demonstrates that BYI 08330-desmethyl-enol was excreted more rapidly than BYI 08330-enol.
- The highest proportions of BYI 08330-desmethyl-enol were detected in liver, where the compound is formed by metabolic transformation of BYI 08330-enol. The proportions in plasma, kidney, and testis were comparable and significantly lower than in liver.
- BYI 08330-ketohydroxy was found as a further prominent metabolite in liver and kidney, but was only present at trace levels in plasma, testis, and urine.

For the <u>high dose tests</u>:

- Absorption and excretion were slower and lower than in low dose tests. Only 27 % of the dose was renally excreted 24 h after dosage.
- The residues in plasma were slightly higher than in liver and kidney. This may be caused by the saturation of the active transport mechanisms at high concentrations resulting in a more uniform distribution of the compound in the body. The decline of residues was slower than in the low dose tests.
- As observed in low dose groups, the residues in testis, carcass and skin were lower than in plasma. Plasma and all organs showed slow depletion of radioactive residues.
- The metabolism was similar to the metabolism of the low dose tests, with the exception that BYI 08330-desmethyl-enol was found at higher proportions in high dose tests.

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- As in low dose tests, BYI 08330-desmethyl-enol was found at higher proportions in urine (ratio BYI 08330-enol / BYI 08330-desmethyl-enol = 1.5: 1) than in plasma and organs (ratios from 5: 1 up to 35 : 1) due to the rapid excretion of this metabolite.
- The highest percentages of BYI 08330-desmethyl-enol were detected in liver as well as in kidney. The percentages in plasma, kidney and testis were lower and comparable.
- Significant percentages of BYI 08330-ketohydroxy were present in liver and kidney only.

Based on all results, it can be concluded that due to the saturation of active transport mechanisms in the excretory organs after administration of high doses, the depletion of residues and the excretion via urine and faeces is slow and with a potential of accumulation in the body following repeated high doses.

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Table 5.1.1-11: Balance of radioactivity in excreta, plasma, organs and tissues of male rats after single oral administration of 2 mg and 1000 mg [azaspirodecenyl-3-^{14}C] BYI 08330/kg bw

	Radioactivity in % of dose administered (mean values)					
Test no.;	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
No. of animals:	4	4	4	4	4	4
Admin.-route:	oral	oral	oral	oral	oral	oral
Dose [mg/kg bw]:	**2**	**2**	**2**	**1000**	**1000**	**1000**
Spec. radioactivity [KBq/mg]	3670	3670	3670	7.34	7.34	7.34
Test duration	1 h	8 h	24 h	1 h	8 h	24 h
Urine						
0 - 1 h	12.5	--	--	1.6	--	--
0 - 4 h	--	20.1	37.3	--	4.9	6.0
4 - 8 h	--	45.6	39.4	--	10.9	9.4
8 – 24 h	--	--	35.3	--	--	11.4
Sum Urine	12.5	65.7	111.9	1.6	15.8	26.8
Faeces	*	*	9.5	*	*	17.8
Erythrocytes	1.4	0.3	0.003	0.3	0.2	0.1
Plasma	3.5	1.0	0.003	0.5	0.4	0.1
Liver	22.9	9.2	0.067	1.2	0.9	0.4
Kidney	4.8	1.5	0.005	0.3	0.2	0.1
Testis	0.4	0.2	0.002	0.1	0.1	<0.1
Sum Organs	33.1	12.3	0.080	2.4	1.8	0.7
Skin	12.7	3.2	0.031	3.0	2.4	0.7
Carcass	28.3	10.6	0.097	5.9	6.1	1.8
GIT	32.1	8.6	0.205	85.9	72.9	47.0
Total Body	106.2	34.6	0.413	97.1	83.1	50.2
Balance	**118.7**	**100.3**	**121.8**	**98.7**	**98.9**	**94.8**

* for 1 h and 8 h animals, the faeces were included in the GIT

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Table 5.1.1-12: Total radioactive residues in organs and tissues of male rats sacrificed 1 h, 8 h, and 24 h after single oral administration of 2 mg and 1000 mg [azaspirodecenyl-3-^{14}C]BYI 08330/kg bw

Test no.;	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
No. of animals:	4	4	4	4	4	4
Admin.-route:	oral	oral	oral	oral	oral	oral
Dose [mg/kg bw]:	**2**	**2**	**2**	**1000**	**1000**	**1000**
Spec. radioactivity [KBq/mg]	3670	3670	3670	7.34	7.34	7.34
Test duration	1 h	8 h	24 h	1 h	8 h	24 h
TRR [mg a.s. equiv./kg] (mean values)						
Erythrocytes	1.723	0.412	0.003	199.2	147.9	47.1
Plasma	4.527	1.242	0.004	352.5	271.4	102.0
Liver	11.740	5.073	0.024	315.2	245.9	109.0
Kidney	11.220	3.517	0.011	307.9	221.7	85.3
Testis	0.668	0.342	0.004	66.5	77.4	28.5
Skin	0.978	0.259	0.003	119.2	93.4	31.7
Carcass	0.906	0.355	0.005	97.8	103.3	33.2
Dose normalised concentration CN (mean values)						
Erythrocytes	0.951	0.220	0.002	0.212	0.156	0.045
Plasma	2.498	0.663	0.003	0.376	0.287	0.098
Liver	6.487	2.701	0.013	0.336	0.260	0.105
Kidney	6.197	1.873	0.006	0.328	0.235	0.082
Testis	0.369	0.182	0.002	0.071	0.082	0.028
Skin	0.540	0.138	0.001	0.127	0.099	0.031
Carcass	0.500	0.190	0.002	0.104	0.110	0.032
Radioactivity in percent of dose administered (mean values)						
Erythrocytes	1.42	0.34	0.003	0.28	0.23	0.06
Plasma	3.52	0.98	0.003	0.52	0.42	0.14
Liver	22.92	9.23	0.067	1.21	0.86	0.38
Kidney	4.83	1.48	0.005	0.26	0.18	0.07
Testis	0.42	0.24	0.002	0.08	0.09	0.04
Skin	12.71	3.22	0.031	2.97	2.37	0.71
Carcass	28.30	10.55	0.097	5.87	6.10	1.81

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Table 5.1.1-13: Metabolite distribution in urines of male rats collected 0 – 1 h, 0 – 8 h and 0 – 24 h after single oral administration of 2 mg and 1000 mg [azaspirodecenyl-3-^{14}C]BYI 08330/kg bw

Test		Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
Dose [mg/kg bw]		2	2	2	1000	1000	1000
Sacrifice [h]		1 h	8 h	24 h	1 h	8 h	24 h
Sampling interval		0 - 1 h	0 - 8 h	0 - 24 h[a]	0 - 1 h	0 - 8 h	0 - 24 h[a]
% of dose administered:		12.5	65.7	111.9	1.6	15.8	26.8
Peak no.	Report name BYI 08330-	**% of administered dose**					
1	unknown	n.d.	n.d.	0.07	n.d.	n.d.	n.d.
2	**-enol-GA**	**0.04**	**0.40**	**0.55**	**n.d.**	**0.12**	**0.25**
3	unknown	n.d.	0.09	0.16	n.d.	n.d.	0.02
4	unknown	n.d.	0.12	0.17	n.d.	n.d.	0.08
5	unknown	n.d.	0.22	0.36	n.d.	n.d.	0.07
6	unknown	n.d.	0.07	0.12	n.d.	n.d.	0.04
7	unknown	n.d.	n.d.	0.07	n.d.	n.d.	0.03
8	unknown	n.d.	0.10	0.13	n.d.	n.d.	n.d.
9	unknown	n.d.	0.13	0.20	n.d.	n.d.	n.d.
10	unknown	n.d.	n.d.	0.02	n.d.	n.d.	n.d.
11	**-enol-alcohol**	**0.26**	**0.81**	**1.15**	**n.d.**	**0.16**	**0.33**
12	**-desmethyl-enol**	**1.26**	**20.50**	**33.09**	**0.15**	**4.18**	**10.63**
13	unknown	0.10	0.78	1.17	n.d.	0.16	0.29
14	**-desmethyl-ketohydroxy**	**n.d.**	**0.13**	**0.20**	**n.d.**	**n.d.**	**0.03**
16	**-enol**	**10.72**	**42.12**	**74.07**	**1.46**	**11.15**	**14.99**
18	**-ketohydroxy**	**0.07**	**0.26**	**0.37**	**n.d.**	**n.d.**	**0.05**
Total:		12.45	65.73	111.90	1.61	15.77	26.81
Identified:		**12.4**	**64.2**	**109.4**	**1.6**	**15.6**	**26.3**
Unknown *)		0.1	1.5	2.5	n.d.	0.2	0.5
Total:		**12.5**	**65.7**	**111.9**	**1.6**	**15.8**	**26.8**

[a] calculated sum of urine pools from 0 – 4 h, 4 – 8 h and 8 – 24 h
*) characterised based on their retention time in HPLC

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Table 5.1.1-14: Total radioactive residues of metabolites in plasma, liver, kidney and testis of male rats sacrificed 1 h and 8 h after a single oral administration of 2 mg [azaspirodecenyl-3-^{14}C]BYI 08330/kg bw

	Plasma		Liver		Kidney		Testis	
Test no. Dose [mg/kg bw] Sacrifice [h]	1 2 1 h	2 2 8 h	1 2 1 h	2 2 8 h	1 2 1 h	2 2 8 h	1 2 1 h	2 2 8 h
TRR [mg a.s. equiv./kg]	**4.527**	**1.242**	**11.740**	**5.073**	**11.220**	**3.517**	**0.668**	**0.342**
Report name BYI 08330-	**% of TRR**							
-enol-GA	**n.d.**	**n.d.**	**0.1**	**0.2**	**0.4**	**0.4**	**0.1**	**n.d.**
-enol-alcohol	**n.d.**	**n.d.**	**0.5**	**0.4**	**0.2**	**n.d.**	**0.2**	**n.d.**
-desmethyl-enol	**2.3**	**3.6**	**7.3**	**6.8**	**2.3**	**3.6**	**2.5**	**3.3**
unknown	n.d.	n.d.	0.7	0.7	0.3	0.3	n.d.	n.d.
-desmethyl-ketohydroxy	**n.d.**	**n.d.**	**1.2**	**0.7**	**0.6**	**0.9**	**n.d.**	**n.d.**
unknown	n.d.	n.d.	0.3	0.2	0.5	0.4	n.d.	n.d.
-enol	**97.1**	**96.4**	**72.1**	**79.1**	**73.3**	**74.4**	**94.8**	**83.8**
unknown	n.d.	n.d.	1.0	0.3	1.7	1.1	n.d.	n.d.
-ketohydroxy	**0.6**	**n.d.**	**13.2**	**8.1**	**20.0**	**18.7**	**2.3**	**2.4**
unknown	n.d.	n.d.	0.1	n.d.	0.3	n.d.	n.d.	n.d.
unknown	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.4
unknown	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.7
Subtotal:	100.0	100.0	96.5	96.5	99.5	99.7	99.9	99.4
Identified:	**100.0**	**100.0**	**94.4**	**95.3**	**96.8**	**97.9**	**99.9**	**89.4**
Unknown:*)	n.d.	n.d.	2.1	1.2	2.7	1.8	n.d.	10.0
Not analysed / solids	--	--	3.5	3.5	0.5	0.3	0.1	0.6
Total:	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**

n.d. = not detected

*) characterised based on their retention time in HPLC

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Table 5.1.1-15: Total radioactive residues of metabolites in plasma, liver, kidney and testis of male rats sacrificed 1 h, 8 h, and 24 h after a single oral administration of 1000 mg [azaspirodecenyl-3-^{14}C]BYI 08330/kg bw

	Plasma			Liver			Kidney			Testis		
Test no.	4	5	6	4	5	6	4	5	6	4	5	6
Dose [mg/kg bw]	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Sacrifice [h]	1 h	8 h	24h	1 h	8 h	24h	1 h	8 h	24h	1 h	8 h	24h
TRR [mg a.s. equiv./kg]	**352.5**	**271.4**	**102.0**	**315.2**	**245.9**	**109.0**	**307.9**	**221.7**	**85.3**	**66.5**	**77.4**	**28.5**
Report name BYI 08330-	% of TRR											
-enol-GA	**n.d.**	**n.d.**	**n.d.**	**0.5**	**1.2**	**1.0**	**0.5**	**1.0**	**1.1**	**n.d.**	**n.d.**	**n.d.**
-enol-alcohol	**n.d.**	**n.d.**	**n.d.**	**0.7**	**0.8**	**n.d.**	**0.4**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
-desmethyl-enol	**3.3**	**7.5**	**6.8**	**11.6**	**14.7**	**13.0**	**4.9**	**13.1**	**11.5**	**2.6**	**6.4**	**8.7**
-desmethyl-ketohydroxy	**n.d.**	**n.d.**	**n.d.**	**1.0**	**1.7**	**1.8**	**1.1**	**3.4**	**3.6**	**n.d.**	**n.d.**	**n.d.**
-enol	**96.7**	**92.5**	**93.2**	**76.4**	**72.0**	**74.1**	**75.4**	**64.9**	**65.9**	**94.9**	**91.7**	**89.3**
unknown	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2	1.3	1.2	n.d.	n.d.	n.d.
-ketohydroxy	**n.d.**	**n.d.**	**n.d.**	**5.5**	**6.8**	**6.6**	**16.3**	**16.0**	**16.2**	**2.2**	**1.4**	**1.1**
Subtotal:	100.0	100.0	100.0	95.6	97.1	96.4	99.7	99.7	99.5	99.7	99.5	99.1
Identified:	**100.0**	**100.0**	**100.0**	**95.6**	**97.1**	**96.4**	**98.6**	**98.5**	**98.4**	**99.7**	**99.5**	**99.1**
Unknown:*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2	1.3	1.2	n.d.	n.d.	n.d.
Not analysed / solids	--	--	--	4.4	2.9	3.6	0.3	0.3	0.5	0.3	0.5	0.9
Total:	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**

n.d. = not detected
*) characterised based on their retention time in HPLC

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Figure 5.1.1-02: Proposed metabolic pathway of BYI 08330 in organs and tissues and excreta of male rats

BYI08830

BYI08330-enol-alcohol

BYI08330-enol

conjugation with glucuronic acid

BYI08330-enol-GA

BYI08330-ketohydroxy

BYI08330-desmethyl-enol

BYI08330-desmethyl-ketohydroxy

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Report:	**KIIA 5.1.1/05, . 2006**
Title:	PBPK-Simulation of BYI 08330 in male rats at high doses
Report No & Document No	BTS-WSM0603-1 M-274847-02-2
Guidelines:	Not applicable
GLP	No (simulation)
Testing Facility and Dates	, Germany Experimental work: Simulation, no experimental study

Executive Summary

In a previous study (Report No. BTS-WSM0602), the pharmacokinetics of BYI 08330 in male rats have been modelled physiologically based. To confirm the predicted behaviour, an additional specifically designed experimental study (Report No. MEF-06/328) was conducted including a dose of 1000 mg/kg in which plasma and tissue concentrations of BYI 08330 metabolites in liver, kidney and testis had been determined. The experimental results confirmed the predictions of the previous simulations.
These most recently obtained experimental data were used to improve the parameterization of the physiologically based pharmacokinetic (PBPK) model developed before. Thus most of the experimental information about plasma and tissue concentrations and urinary excretion of BYI 08330-enol as well as BYI 08330-desmethyl-enol could be well described by the refined model. The major difference to the former model was the significant saturation of the active uptake into the liver which was underestimated before due to a lack of information. Liver and kidney tissue concentrations of the secondary metabolite BYI 08330-desmethy-enol revealed the presence of active uptake processes in these tissues for this substance, too.
The PBPK model with the parameter setting improved according to the new experimental data was used again to simulate the pharmacokinetics in male rats after repeated administration of various doses over 28 days. The following predictions were obtained from the calculations:

- Average daily plasma concentrations of BYI 08330-enol remain on a constant level from day to day at daily doses below 300 mg/kg and rise strongly with the time at daily doses above 300 mg/kg.
- At a daily dose of 300 mg/kg, a moderate, twofold increase of plasma concentration time curves is predicted within the first 14 days until a steady state is reached.
- At doses of 500 mg/kg and 1000 mg/kg, the peak concentration at day 28 is 5.5 and 8.1 times larger than that of the first day and rises further with prolonged administration. This results in a distinct, disproportionate increase of the body burden by BYI 08330-enol.
- The prospective behaviour of the secondary metabolite BYI 08330-desmethyl-enol shows an even more pronounced change at very high doses. The dose normalized plasma concentrations are stable at low levels up to 500 mg/kg but rise 100-fold with the time at 1000 mg/kg due to saturation of active transport processes also for this substance.

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Overall the PBPK simulations demonstrated that repeated daily doses of 300 mg BYI 08330/kg bw and higher will lead to non-linear elimination kinetics, resulting in a high body burden in multiple-dose toxicological studies.

I. Material and Methods

A. Material

No test material was used since this report includes a simulation and not an experimental study.

B. Study Design

Pharmacokinetic simulation

Physiology based pharmacokinetic simulations were carried out with the commercially available software PK-Sim (Bayer Technology Services GmbH).

The simulation used the physiological parameters optimized in the first PBPK simulation (Report No. BTS-WSM0602) which were refined to match the results of the plasma and organs metabolism investigations for low and high dose levels (Report No. MEF-06/328).

The following parameters were calculated in the new simulation:

- Time course for plasma and organ concentrations of metabolite BYI 08330-enol after a single dose of 2mg/kg bw and 1000 mg/kg bw
- Time course for plasma and organ concentrations of metabolite BYI 08330-desmethyl-enol after a single dose of 2mg/kg bw and 1000 mg/kg bw
- Time course for fractions of renal excretion for metabolites BYI 08330-enol and BYI 08330-desmethyl-enol after a single dose of 2mg/kg bw and 1000 mg/kg bw
- Time course for plasma concentrations of metabolites BYI 08330-enol and BYI 08330-desmethyl-enol after repeated daily administration of BYI 08330 for 4 weeks. The dose range covered was 10 – 1000 mg/kg bw.

II. Results and Discussion

For BYI 08330-enol, the new adjustment of model parameters had only minor influence on the simulation results at low doses. Thus at the dose of 2 mg/kg the correlation between simulation results and observed data remained very well comparable to the results of the first PBPK simulation (Report No. BTS-WSM0602). For the high dosage (1000 mg/kg), the calculated concentrations were about sevenfold too high compared to the observed values. However, other important results as liver, kidney and testis tissue/plasma partition coefficients 8 h after administration, the fractions of administered dose excreted in urine and the peak/trough ratios for both dosage regimens of the experimental study were as well represented by the simulation of 1000 mg/kg as by that of 2 mg/kg.

Simulation results for BYI 08330-desmethyl-enol after administration of 2 mg/kg and 1000 mg/kg BYI 08330 respectively in comparison to the observed concentrations in plasma, liver, kidney and testis were of lower quality. Regarding the sparse experimental information and the fact that the simulation is based on the output of another simulation the observed data points are described satisfactorily by the calculation.

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In addition to the systemic concentrations, the fractions of dose which were excreted in urine as BYI 08330-enol and BYI 08330-desmethyl-enol were calculated in the simulations and were compared to the respective experimental values. Again the observed behaviour was well reflected by the simulation.

The plasma concentration time curve of the BYI 08330-enol after multiple applications was confirmed by this refined PBPK-modelling. Mean daily plasma concentrations were kept constant over time after doses of 10 and 100 mg/kg bw/day. Due to the beginning of the saturation of the uptake process into liver and possibly also kidney cells the transition from stable to rising plasma concentrations is starting at a daily dose of 300 mg/kg bw/day and is already fully developed at 500 mg/kg as is shown in the dose dependence of dose normalized C_{max} and AUC and of the ratio $C_{max(day1)}/C_{max(day28)}$ listed below.

Daily dose [mg/kg]	AUC_{norm} [kg.h/l]	C_{max_norm} [kg/ml]	$C_{max(day\ 28)}/C_{max(day\ 1)}$
10	240	0.0022	1.0
100	662	0.0034	1.0
300	2933	0.0069	1.9
500	7066	0.0172	5.5
1000	6789	0.0168	8.1

III. Conclusions

Using the most recent experimental information about tissue concentrations in liver and kidney in the high dose regimen and the concentrations of the metabolite desmethyl-enol (Report No. MEF-06/328) the parameterization of the PBPK-model for BYI 08330 could significantly be improved. The results of the PBPK-simulations showed a good to fair agreement with observed data for the time course of the plasma and tissue concentrations of BYI 08330-enol and BYI 08330-desmethyl-enol as well as for the excreted amounts. In most cases the calculated data show deviations from those observed within or below a factor of two. For the dose of 1000 mg/kg, the calculated enol concentrations are 4- to 7-fold higher than those determined experimentally. No explanation could be found for this discrepancy, although several hypotheses were tested. However, other results that depend on the BYI 08330-enol plasma concentration, (e.g. the excreted amounts of BYI 08330-enol and BYI 08330-desmethyl-enol or the BYI 08330-desmethyl-enol plasma and tissue concentrations) are in good agreement with the respective experimental data.

Moreover it could be shown that the saturation of the transport processes involved in the excretion is predominantly responsible for the decreases of the peak/trough ratio at high doses which was experimentally confirmed for 1000 mg/kg. For the application of the model in simulations of scenarios not investigated experimentally it is, however, important that despite of the discrepancy in the absolute concentrations, found at high doses, the general pharmacokinetic behaviour with the strong reduction of $C_{max}/C_{(24h)}$ with increasing dose is well represented by the calculations. The reduction of the peak/trough ratio is the main reason for the strong rise of concentrations predicted for repeated administration of doses larger than 300 mg/kg.

Report:	**KIIA 5.1.1/06, [redacted].; 2006**
Title:	[Azaspirodecenyl-3-^{14}C]-BYI 08330: Comparison of the in vitro metabolism in Liverbeads™ from male rat, mouse and human
Report No & Document No	SA 05319 M-274118-01-2
Guidelines:	no written test guideline available for this type of study
GLP	Yes, according to O.E.C.D. Principles of Good Laboratory Practice, 1997 (January 26,1998). European guideline 2004/10/EC (February 11, 2004). French Decree N°98 1312, on December 31, 1998 regarding Good Laboratory Practice. E.P.A. (Environmental Protection Agency) 40 CFR part 160 Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards: Final Rule, August 17, 1989. Good Laboratory Practice Standards for Toxicology studies on Agricultural chemicals, Ministry of Agriculture, Forestry and Fisheries (M.A.F.F.), notification 11 Nousan n° 6283, October 01, 1999 modified by 12 Nousan n° 8628, December 06, 2000.
Testing Facility and Dates	[redacted] [redacted], France Experimental work: December 14, 2005 – January 11, 2006

Executive Summary

Liver cells (Liverbeads™) from male rat, mouse, and human were incubated with [azaspirodecenyl-3-^{14}C] BYI 08330, during 4 hours at the final concentration of approximately 50µM and 520µM.

The metabolites, which were formed were quantified by HPLC with radiodetection and identified by HPLC-MS.

At high concentration, the enzymatic capabilities of the in vitro system seemed to be saturated and therefore no supplementary information was obtained.

The results of the low concentration test can be summarized as follow:

In the liver cells from <u>all species</u>, BYI 08330 was completely metabolized and no parent compound was detected at the end of the incubation. BYI 08330-enol was the first and most prominent metabolite accounting for 66 – 92 % of total metabolites.

Marked differences occurred in the metabolic profile between the three species at low concentration:

- In the <u>rat</u>, the BYI 08330-enol was further metabolised by oxidation reactions to BYI 08330-desmethyl-enol (oxidative demethylation), BYI 08330-enol-alcohol (oxidation of aromatic methyl group) and BYI 08330-ketohydroxy (oxidation of the azaspirodecenyl moiety). Oxidation products accounted for ca. 14 %. Conjugation was not detected as an in vitro metabolic transformation

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- The general picture of the "in vitro" metabolic pathway in the rat was very similar to the in vivo metabolism in the male rat. The same metabolites were detected with BYI 08330-enol and BYI 08330-desmethyl-enol as the two most important degradation products although the proportions of metabolites were different between in vivo and in vitro tests.
- In the mouse, oxidative degradation of BYI 08330-enol was detected as a minor "in vitro" metabolic reaction, only (4 % of oxidation products). Conjugation to BYI 08330-enol-GA was very prominent with the conjugate accounting for ca. 30 %.
- Human liver cells exhibited an "in vitro" metabolism more similar to the one in mouse than in the rat. Conjugation to BYI 08330-enol-GA (6 %) was more prominent than oxidative transformation which was detected as a minor transformation (1 %), only.

I. Material and Methods

A. Test Material

IUPAC Name:	**cis-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate**	
Code name:	**BYI 08330**	
Common name:	**Spirotetramat (proposed ISO)**	
Empirical formula:	$C_{21}H_{27}NO_5$	
Molar mass:	**373.45 g/mol**	
Water solubility:	**pH 4 = 33.5 mg/L, pH 7 = 29.9 mg/L pH 9 = 19.1 mg/L (unstable) all at 20°C**	
n-Octanol/water partition coefficient:	**pH 4 = 2.51, pH 7 = 2.51, pH 9 = 2.50**	
Labelling:	**azaspirodecenyl-3-^{14}C**	
Specific radioactivity of the original radiolabelled batches:	**3.67 MBq/mg (99.1 µCi/mg)**	
Specific radioactivity used for administration:	**Low concentration assay:**	**3.67 MBq/mg (99.1 µCi/mg)**
	High concentration assay:	**1.84 MBq/mg (46 µCi/mg)**
	radiodilution 1 / 1 with non radiolabelled test compound	
Radiochemical purity:	**> 98 %**	
Dose level:	**Low concentration assay:**	**50 µM**
	High concentration assay:	**520 µM**
Stability of the test material:	**The stability of [azaspirodecenyl-3-^{14}C]BYI 08330 was demonstrated by radio HPLC analysis of the stock solution before incubation**	

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B. Test System

Liverbeads™ from male rat, mouse and human

Liverbeads™ are immobilized hepatocytes entrapped within an alginate matrix. They were purchased from , France. These hepatocytes were used in the incubation assays to study the in vitro metabolism of BYI 08330:

- Rat: Wistar male rat, batch LVB008001.
- Mouse: CD1 male mice, batch LVB002005
- Human: male, batch LVB005005

C. Study Design

In vitro metabolism of BYI 08330 by Liverbeads™ from rat, mouse, and human

Liverbeads™ were thawed according to the procedures described by the supplier. Then, the cells were pooled in HBSS (Hank's balanced salt solution) plus penicillin 50 UI/ml and streptomycin 50 µg/ml plus glucose (25 mM).

The Liverbeads™ were then seeded in 12 well plates, one plate per concentration. The enzymatic reactions were initiated by the addition of the radiolabelled product in acetonitrile at the final concentration of approximately 50 µM or 520 µM. Control samples (Liverbeads + acetonitrile) were also incubated without $[^{14}C]$BYI 08330. Then, the plates were placed in an incubator at 37°C under gentle shaking. Each assay (test) was run in duplicate. The time period of incubation was 4 hours.

At the end of the incubation time, the Liverbeads™ were first dissolved using EDTA Na_2 (100mM), and then the hepatocytes were sonicated during 20 sec. The supernatants including hepatocytes plus incubation medium plus dissolved Liverbeads™ from each well were then transferred into separated vials and immediately stored at -20°C until LC/MS analysis.

Metabolite analysis

Hepatocytes incubations were centrifuged and the supernatants were directly injected into the HPLC coupled with a UV detector, ^{14}C detector, and a mass spectrometer. A C18 column with the solvents 0.1 % aqueous acetic acid / acetonitrile and gradient elution was used for quantification of the metabolites by integration of the ^{14}C signal. The calculated area from each peak was then expressed as percentage of the sum of the areas from the different peaks (corresponding to metabolites).

Metabolites were identified by their mass spectra measured in electrospray positive mode. Identification of some metabolites was supported by comparison of retention times and spectra with those of authentic reference compounds.

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II. Results and Discussion

This study was performed to compare the metabolic profile of BYI 08330 between different species using an in vitro system. The test system was based on Liverbeads™ from male rat, mouse and human which were incubated during 4 hours with [azaspirodecenyl-3-14C]-BYI 08330 at the concentration of 50 µM and 520 µM.

At low concentration, analysis of the samples using LC/MS demonstrated the presence of five different metabolites in the mouse, four in the rat and three in the human Liverbeads™. The parent compound BYI 08330 was not detected in the Liverbeads™ from any tested species.

The major metabolites detected in the mouse were BYI 08330-enol and BYI 08330-enol-GA (enol glucuronide) which occurred at 66 % and 30 % respectively. The glucurono-conjugation of the main metabolite BYI 08330-enol appeared to be a major route in the in vitro degradation (detoxification) of BYI 08330 in the mouse. BYI 08330-enol-alcohol, BYI 08330-desmethyl-enol and BYI 08330-ketohydroxy were detected at a very low level in the mouse (1-2 %).

In the rat, the main metabolites were BYI 08330-enol and BYI 08330-desmethyl-enol which occurred at 87 % and 7 % respectively. BYI 08330-enol-alcohol and BYI 08330-ketohydroxy represented 4 % and 3 % respectively. The BYI 08330-enol-GA (enol glucuronide) was not detected in vitro in the rat.

The major metabolites detected in human were BYI 08330-enol and BYI 08330-enol-GA (enol glucuronide), but their relative abundance were different from those observed in the mouse. They occurred at 92 % and 6 % respectively. BYI 08330-desmethyl-enol was present at a very low level (1 %), BYI 08330-enol-alcohol and BYI 08330-ketohydroxy were not detected in human.

At high concentration, no new metabolite was detected in the rat, mouse and human Liverbeads™. The low degree of metabolism observed in this experiment could be indicative of a saturation of the biotransformation enzymatic system. The results of the individual tests are shown in Table 5.1.1-16.

The metabolic pathway for the in vitro test system is presented in Figure 5.1.1-03

III. Conclusion

Liverbeads™ from male rat, mouse, and human were incubated with [azaspirodecenyl-3-^{14}C] BYI 08330, during 4 hours at the final concentration of approximately 50µM and 520µM.

At high concentration, the enzymatic capabilities of the in vitro system seemed to be saturated therefore no supplementary information was obtained.

The results of the low concentration test can be summarized as follow:

In the liver cells from all species, BYI 08330 was completely metabolized and no parent compound was detected at the end of the incubation. BYI 08330-enol was the first and most prominent metabolite accounting for 66 – 92 % of total metabolites.

Marked differences occurred in the metabolic profile between the three species at low concentration:

- In the rat, the BYI 08330-enol was further metabolised by oxidation reactions to BYI 08330-desmethyl-enol (oxidative demethylation), BYI 08330-enol-alcohol (oxidation of aromatic methyl group) and BYI 08330-ketohydroxy (oxidation of the azaspirodecenyl moiety). Oxidation products accounted for ca. 14 %. Conjugation was not detected as an in vitro metabolic transformation
- The general picture of the "in vitro" metabolic pathway in the rat was very similar to the in vivo metabolism in the male rat. The same metabolites were detected with BYI 08330-enol and

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BYI 08330-desmethyl-enol as the two most important degradation products although the proportions of metabolites were different between the in vivo and in vitro tests.

- In the <u>mouse</u>, oxidative degradation of BYI 08330-enol was detected as a minor "in vitro" metabolic reaction, only (4 % of oxidation products). Conjugation to BYI 08330-enol-GA was very prominent with the conjugate accounting for ca. 30 %.
- <u>Human</u> liver cells exhibited an "in vitro" metabolism more similar to the one in mouse than in the rat. Conjugation to BYI 08330-enol-GA (6 %) was more prominent than oxidative transformation which was detected as a minor transformation (1 %), only.

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Table 5.1.1-16: Distribution of the parent compound and metabolites after incubation of BYI 08330 to Liverbeads of rat, mouse, and human

Low concentration experiment: distribution of parent compound and metabolites (sum = 100)						
	enol-GA (glucuronide) M=477	enol-alcohol M=317	desmethyl-enol M=287	enol M=301	keto hydroxy M=317	BYI 08330
rat	0	4	7	87	3	0
mouse	30	1	1	66	2	0
human	6	0	1	92	0	0
High concentration experiment: distribution of parent compound and metabolites (sum = 100)						
rat	0	0	0	100	0	0
mouse	9	1	0	89	1	0
human	2	0	0	98	0	0

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Figure 5.1.1-03: In vitro metabolic pathway of BYI 08330 in Liverbeads™ from male rat, mouse, and human

BYI08330

Rat
Mouse
Human

BYI08330-enol

Mouse
Rat

BYI08330-enol-alcohol

Human
Mouse

BYI08330-enol-GA

Mouse
Rat

BYI08330-keto-hydroxy

Rat
Mouse
Human

BYI08330-desmethyl-enol

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Toxicokinetic studies with metabolites- single dose, oral route, in rats

Report:	**KIIA 5.1.1/07, 2006**
Title:	[Azaspirodecenyl-3-14C]BYI 08330-enol-glucoside supplemental Study: Adsorption, Distribution, Excretion and Metabolism in the Rat
Report No & Document No	MEF 06/006 M-268645-01-2
Guidelines:	US EPA Guideline No.: OPPTS 870.7485 Metabolism and Pharmacokinetics, EPA Ref.: 712-C-98-244, August 1998 EU Council Directive 91/414/EEC amended by the Commission Directive 94/79/EC, adopted December 21, 1994 OECD Guideline for Testing Chemicals, 417: Toxicokinetics adopted April 04, 1984 Japanese MAFF New Test Guidelines for Supporting Registration of Chemical Pesticides, 12 Nousan 8147, adopted November 24, 2000, amended June 26, 2001
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	, Germany Experimental work: 2004-04-25 – 2004-09-21

Executive Summary

BYI 08330-enol-glucoside was a main metabolite of BYI 08330 in the lettuce metabolism study and hence included in the plant residue method. BYI 08330-enol-glucoside was detected in fruiting and leafy crops of European residue trials (unpublished results). Because it was not detected in the rat ADME-study, an additional ADME-study with this compound was conducted in order to investigate the absorption, distribution, excretion and metabolism of BYI 08330-enol-glucoside in male rats.

One male rat was administered by oral gavage with a single dose of BYI 08330-enol-glucoside in aqueous saline solution at a target dose level of 0.1 mg/kg body weight. The test item was radiolabelled with ^{14}C in the 3-position of the spiro-ring of the molecule (azaspirodecenyl-3-^{14}C label).

Microplasma, urine and faeces were collected at various times of post administration. Skin, gastro-intestinal tract and carcass were sampled at sacrifice.

98.3 % of the administered dose were recovered from measurement of the total radioactivity in urine and faeces as well as in skin, GIT and carcass.

The radiolabelled BYI 08330-enol-glucoside was rapidly absorbed from the gastrointestinal tract of the male rat. The absorption commenced immediately after oral dosing. About half the dose was absorbed as derived from the rate of renal excretion (53.3 %) and the residue in the body without GIT (1.07 %) at the time of sacrifice 48 h after dosing.

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The distribution of the test substance from the central compartment to the different organs and tissues was followed by measuring the concentration of the total radioactivity in plasma. Plasma radioactivity peaked ca. 4 h after dosing at a level of 0.0232 mg eq./kg bw (values calculated with the pharmacokinetic software TOPFIT). From the maximum value, the levels slowly declined by a factor of ca. 30 x at 24 h down to levels < 0.0001 at 48 h after treatment.

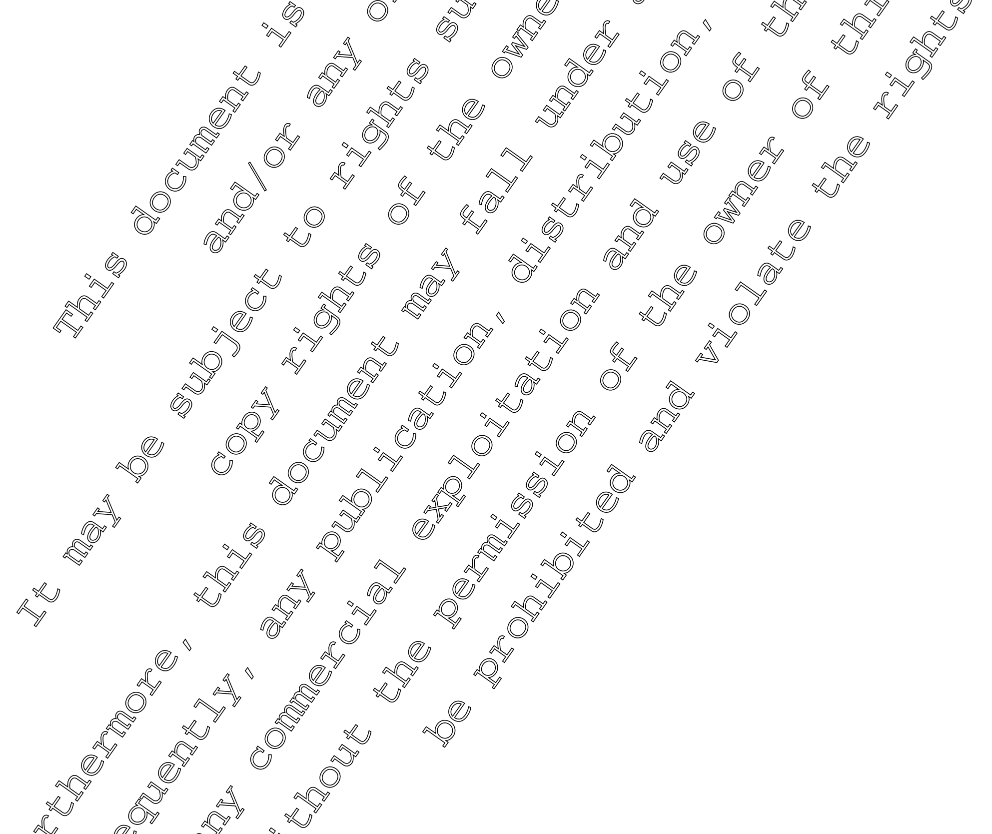
The radiolabelled residues in skin, GIT and carcass of the animal were determined at sacrifice, 48 h after dosage. Negligible amount of radioactivity was found in skin (0.09 %) and GIT (0.11 %). The radioactivity in the carcass amounted to 1 % of the dose.

Excretion was fast and almost complete 24 h after administration. From the total excreted amount of 97.1 %, 53.3 % and 43.7 % of the dose were excreted with urine and faeces, respectively.

The test item and metabolites were quantified and identified by reversed phase radio-HPLC. The identification rate was high and amounted to 93 % of the administered dose. BYI 08330-enol was the main metabolite in excreta amounting to about 64 % of the dose. Minor metabolites were BYI 08330-desmethyl-enol with about 5 % and BYI 08330-ketohydroxy with about 3 % of the dose.

The unchanged BYI 08330-enol-glucoside was detected in excreta with about 21 % of the dose, whereof 20.7 % of the dose were found in faeces.

All metabolites detected in this study were identical with the metabolites identified in the ADME study with the new insecticide BYI 08330.



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I. Material and Methods

A. Material

1. Test Material

IUPAC Name:	**(5s,8s)-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl D-glucopyranoside**
Code name:	**BYI 08330-enol-glucoside**
Common name:	**Does not apply**
Empirical formula:	**$C_{24} H_{33} N O_8$**
Molar mass:	**463.53 g/mol**
Water solubility:	**unknown**
n-Octanol/water partition coefficient:	**unknown**
Labelling:	**azaspirodecenyl-3-^{14}C**
Specific radioactivity of the original radiolabelled batches:	**2.96 MBq/mg or 177,600 dpm/µg**
Specific radioactivity used for administration:	**2.96 MBq/mg or 177,600 dpm/µg**
Radiochemical purity:	**> 99 % (HPLC with radiodetection)**
Dose level:	**0.1 mg/kg body weight**
Vehicle:	**Aqueous saline (0.9 % NaCl in water adjusted to pH 5)**
Stability of the test material:	**The stability of [azaspirodecenyl-3-^{14}C] BYI 08330-enol-glucoside was demonstrated by radio HPLC analysis of the administration solution immediately after dosing**

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2. Test Animals

Species:	**Rat (*Rattus norvegicus domesticus*)**
Strain:	**Wistar Hsd/Cpb: WU**
Breeding facility:	**, Germany**
Sex and numbers involved:	**Males: 1**
Age:	**8 weeks at the time of delivery**
Body weight:	**Males: 199 g at the time of administration** **202 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 23 - 25 °C, relative humidity 40 – 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 , 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***

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B. Study Design

Dosing

The male rat orally received ca. 0.1 mg / kg bw of azaspirodecenyl-3-^{14}C labelled BYI 08330-enol-glucoside dissolved in aqueous saline after 16 hr fasting. The dosing solution was prepared one day before dosing and stored at 4°C. The radiolabelled test item administered in this study was isolated from plant material in the BYI 08330 lettuce metabolism study [███████████, 2004, Report MEF-049/04], purified by HPLC and identified by spectroscopic methods. The sample code of the purified metabolite was BD1120D. The solution was administered to the rat by oral gavage using a syringe attached to an animal-feeding knob cannula. The animal received a volume of 2 mL (= 10 mL/kg bw calculated for a nominal body weight of 200 g). The concentration of the administration solution was calculated to reach an administered amount of about 0.1 mg of the parent compound per kg body weight (bw). The actual administered dose was measured by LSC assay of aliquots of the administration solution and amounted to 0.063 mg [azaspirodecenyl-3-^{14}C]BYI 08330-enol-glucoside / kg bw. The stability of the test compound in the solution was determined by radio HPLC analysis of aliquots after administration.

Collection of blood

Blood samples were collected by pressing a capillary coated with heparin in a small cut in the tail vein. The wound was closed with adhesive tape. The capillaries were centrifuged at ca. 12000 g for about 10 minutes using a hematocrit centrifuge to separate the plasma from the formed constituents (mainly erythrocytes). After centrifugation, the capillary was broken at the border between plasma and formed constituents and the plasma (ca. 30 µL) was pressed onto a small metal dish for weighing. The dish was then placed into a scintillation vial for radioactivity measurement.

Collection of excreta

Urine was collected at various times in a cryogenic trap cooled with dry ice. The funnel for urine collection was 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. Urine samples were stored in a freezer before analysis.
Faeces were collected at various times separately in a cryogenic trap before they were lyophilised (freeze-dried), weighed, and homogenised. The radioactivity was determined by combustion/LSC. Lyophilised samples were stored at room temperature before extraction/analysis.

Sacrifice and organ/tissue sampling

At the respective test end (48 h after administration), the animals were anaesthetised using Pentobarbital-Na. They were sacrificed by transection of the cervical blood vessels. The following organs/tissues were collected: gastro-intestinal tract (GIT), skin, and carcass.
The organs and tissues prepared at the end of the experiments were weighed immediately after the dissection and again following lyophilisation. The radioactivity remaining in the GIT, skin, and residual carcass was determined by combustion/LSC in order to establish the radioactivity balance..

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Radioactivity measurement

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). For this purpose, the volume of the entire solution was determined and radioactivity of an aliquot (1 – 3 replicates) of the solution was measured:

Solid samples (1 – 5 aliquots) were weighed and combusted in an oxygen atmosphere using the following equipments Oxidizer 307/387 (Packard Instruments) for combustion of faeces, erythrocytes, lyophilised organs and tissues like e.g. spleen, liver, lung, bone muscle, gastrointestinal tract (GIT), residual carcass and solids (e.g. from faeces after extraction). Carbosorb E (9 mL) was used to trap the combustion product CO_2 and Permafluor E+ (10 mL, Packard Instruments) as scintillator for LSC.

Metabolite analysis

Urine samples were analysed without sample preparation. Radioactivity in the freeze-dried faeces sample for the time range from 0 - 24 h post administration was determined by combustion/LSC. An aliquot of the sample was extracted 3 times with acetonitrile / water (8 : 2, v/v) using a Polytron homogeniser. Each extract was collected by centrifugation and its volume measured. Aliquots of each extract were radioassayed by LSC. The three extracts were combined, concentrated to the aqueous remainder and partitioned 3 times against n-hexane. Aliquots of aqueous and organic phases were radioassayed by LSC. The aqueous phase was concentrated and submitted for HPLC analysis.

The metabolites in the samples were quantified by HPLC using a reversed phase column (C18) and the eluting solvents water/formic acid = 99 : 1 (v/v) and acetonitrile/water/formic acid = 97 : 2 : 1 (v/v/v) in the gradient mode. Detection was performed by a UV (254 nm) and a radioisotope detector with a glass scintillator.

Metabolites in urine and extracts of faeces were identified by comparison of HPLC profiles of samples with those from the BYI 08330 ADME rat study (Report No. MEF-048/04) and by co-chromatography with reference compounds.

II. Results and Discussion

A. Absorption

The radiolabelled BYI 08330-enol-glucoside was rapidly absorbed from the gastrointestinal tract of the male rat. The absorption commenced immediately after oral dosing as shown by the plasma curve and the calculated absorption half-life of 2.94 hours. About half the dose was absorbed as derived from the rate of renal excretion (53.3 %) and the residue in the body without GIT (1.07 %) at the time of sacrifice 48 h after dosing.

B. Distribution

The distribution of the test substance from the central compartment to the different organs and tissues was followed by measuring the concentration of the total radioactivity in plasma. Plasma radioactivity peaked ca. 4 h after dosing at a level of 0.0232 mg eq./kg bw (values calculated with the pharmacokinetic software TOPFIT). From the maximum value, the levels slowly declined by a factor of ca. 30 x at 24 h down to levels < 0.0001 at 48 h after treatment.

The radiolabelled residues in skin, GIT and carcass of the animal were determined at sacrifice, 48 h after dosage. Negligible amount of radioactivity was found in skin (0.09 %) and GIT (0.11 %). The radioactivity in the carcass amounted to 1 % of the dose.

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C. Metabolism

BYI 08330-enol was the main metabolite in excreta accounting for about 64 % of the dose administered. Minor metabolites were BYI 08330-desmethyl-enol with about 5 % and BYI 08330-ketohydroxy with about 3 % of the dose. The unchanged BYI 08330-enol-glucoside was detected in excreta with about 21 % of the dose, whereof 20.7 % of the dose were found in faeces. In total, ca. 93 % of the administered dose could be identified. The distribution of metabolites in excreta is presented in Table 5.1.1-18. The proposed metabolic pathway of BYI 08330-enol-glc in the rat is shown in Figure 5.1.1-04

D. Excretion

Excretion was fast and almost complete 24 h after administration. From the total excreted amount of 97.1 %, 53.3 % and 43.7 % of the dose were excreted with urine and faeces, respectively.
The excretion is summarized in Table 5.1.1-17.

III. Conclusions

The fate of radio labelled BYI 08330-enol-glc in the male rat following a single oral administration was characterized as follows:

- **Absorption**:
  Fast absorption of the radioactive test compound from the gastrointestinal at a rate of ca. 50 %
- **Distribution**:
  Steady decline of radioactivity concentrations in plasma by several orders of magnitude within 48 hours
- **Excretion**:
  Rapid and complete excretion of radioactivity *via* the renal and faecal route in about the same proportion
- **Metabolism**:
  Cleavage of the glucoside bond resulting in BYI 08330-enol as the major metabolite and key intermediate in the metabolic pathway
- Oxidation of the azaspirodecenyl moiety to BYI 08330-ketohydroxy and demethylation to BYI 08330-desmethyl-enol as minor metabolic reactions
- The metabolism of BYI 08330-enol-glc implies the same pathways as the one of the aglycone BYI 08330-enol and the new insecticide BYI 08330.

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Table 5.1.1-17: Time course of the excretion of radioactivity via urine and faeces of the male rat following a single oral administration of 0.1 mg [azaspirodecenyl-3-^{14}C] BYI 08330-enol-glucoside/kg bw

Cumulative excretion of radioactivity in % of dose administered

Time [h] post admin.	Animal no. 912
Urine	
4	0.7
8	21.5
24	52.5
48	53.3
Faeces	
24	42.7
48	43.7
Sum	97.1

Table 5.1.1-18: Metabolite distribution in urine and faeces of the male rat dosed with [azaspirodecenyl-3-^{14}C]BYI 08330-enol-glucoside

		% of administered dose		
		urine	**faeces**	**Sum**
Time intervals [h]		0-24 h	0-24h	urine + faeces
% of dose analysed		52.5	40.9	93.4
Peak ID HPLC	**Report name**			
Reg 2	BYI 08330-enol-glucoside	0.5	20.7	21.2
Reg 3	BYI 08330-desmethyl-enol	4.6	0.6	5.2
Reg 4	BYI 08330-enol	47.4	16.1	63.5
Reg 5	BYI 08330-ketohydroxy	---	3.1	3.1
Identified		**52.5**	**40.4**	**92.9**
Reg 1	unknown	---	0.5	0.5
Total analysed		**52.5**	**40.9**	**93.4**
Not analysed (urine + faeces 24-48 h)		0.8	1.1	1.9
Solids (from faeces extraction)			1.2	1.2
Organic phase (from faeces extraction)			0.6	0.6
Total in excreta		**53.3**	**43.8**	**97.1**

--- = not detected

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Figure 5.1.1-04 Proposed metabolic pathway of BYI 08330-enol-glucoside in the rat

BYI08330-enol-Glc

BYI08330-enol

BYI08330-ketohydroxy

BYI08330-desmethyl-enol

Report:	**KIIA 5.1.1/08, [redacted] 2006**
Title:	[Azaspirodecane-3-^{14}C]BYI 08330-ketohydroxy: Adsorption, Distribution, Excretion and Metabolism in the Rat
Report No & Document No	MEF-06/007 M-268931-01-2

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Guidelines:	US EPA Guideline No.: OPPTS 870.7485 Metabolism and Pharmacokinetics, EPA Ref.: 712-C-98-244, August 1998 EU Council Directive 91/414/EEC amended by the Commission Directive 94/79/EC, adopted December 21, 1994 OECD Guideline for Testing Chemicals, 417: Toxicokinetics adopted April 04, 1984 Japanese MAFF New Test Guidelines for Supporting Registration of Chemical Pesticides, 12 Nousan 8147, adopted November 24, 2000, amended June 26, 2001
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] [redacted], Germany Experimental work: 2004-10-21 – 2005-06-01

Executive Summary

The test item BYI 08330-ketohydroxy is a metabolite of BYI 08330 and was found in the target crop metabolism studies (MEF-236/04 and MEF-05/288), where it was a main metabolite in cotton. Furthermore, BYI 08330-ketohydroxy was detected in the confined rotational crop study, where it was the main metabolite in Swiss chard and turnip roots and a main intermediate in the metabolic pathway. BYI 08330-ketohydroxy was detected as very minor metabolite only in the urine of the rat ADME study with BYI 08330. Therefore, a supplemental study was conducted to investigate the ADME behaviour of this plant metabolite in the rat.

A group of 4 male rats was administered by oral gavage with a single dose of [azaspirodecenyl-3-^{14}C] BYI 08330-ketohydroxy in aqueous Tragacanth® at a target dose level of 2 mg/kg body weight. The animals were sacrificed 2 days after dosing. About 99 % of the administered dose were recovered from measurement of the total radioactivity in urine and faeces as well as in organs and tissues at sacrifice.

[Azaspirodecenyl-3-^{14}C]BYI 08330-ketohydroxy was rapidly absorbed from the gastrointestinal tract of male rats. The absorption commenced immediately after dosing. The absorption rate was at least 55 % of the total recovered radioactivity, calculated from the values of the urine and the body without the gastrointestinal tract.

Radioactivity in plasma of male rats following a single administration of 2 mg/kg bw reached its calculated maximum at 0.81 h postdose with a calculated maximum radioactivity concentrations in plasma of 1.26 mg eq./kg bw. The peak concentration was slightly below the equal distribution concentration of 2 mg/kg bw BYI 08330-ketohydroxy. Absorption was followed by a fast elimination phase.

The distribution of radioactive residues in the body was analyzed at the time of sacrifice 48 h after dosing by radioactivity measurement of the major organs and tissues. Less than 0.2 % of the

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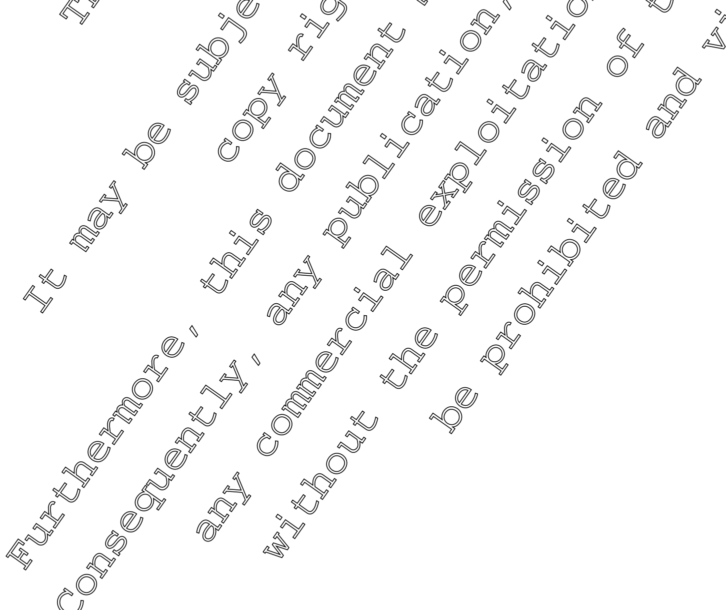
administered dose was detected in the body. Radioactivity concentrations were generally low with the highest levels detected in the gastro-intestinal tract, liver and kidney as the major organs responsible for metabolism and excretion.
The excretion of BYI 08330-ketohydroy was fast and almost completed 24 h after administration. About 54 % of the administered dose was eliminated via urine and about 44 % via faeces within 48 h after dosage.

BYI 08330-ketohydroxy was completely metabolised under formation of numerous metabolites. The parent compound was not detected in urine, traces were found in faeces.
The first and most important metabolic reaction was the oxidative demethylation of the cyclohexyl-O-methyl group to the respective alcohol (BYI 08330-desmethyl-ketohydroxy). Two isomers of BYI 08330-desmethyl-ketohydroxy were detected as the main components in excreta accounting for 15 % of the dose. All other identified metabolites could be derived from BYI 08330-desmethyl-ketohydroxy as an intermediate. Most of the identified compounds were different mono-, di- and tri-oxygenated metabolites which are probably represented by isomers of mono-hydroxy, di-hydroxy and tri-hydroxy metabolites. A second oxidative transformation was formal loss of two hydrogen atoms of the oxygenated metabolites to “dehydro” derivates. These metabolites probably contain aldehyde or keto groups or carboxylic acid groups. LC-MS/MS clearly proved the metabolic transformations but did not allow identifying the positions of oxygenation and the formed functional groups.
The oxygenated metabolites were classified in three groups in order to simplify evaluation and quantification: mono-hydroxy, di-hydroxy and tri-hydroxy metabolites of BYI 08330-desmethyl-ketohydroxy.
About 34 % of the dose was covered by various different mono-hydroxy metabolites and 28 % by the group of di-hydroxy metabolites of BYI 08330-desmethyl-ketohydroxy. Tri-hydroxy metabolites and metabolites formed by cleavage of the azaspirodecenyl ring were of minor importance.
In total, ca. 86 % of the administered dose was identified and ca. 9 % characterised.



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I. Material and Methods

A. Material

1. Test Material

IUPAC Name:	**(5s,8s)-3-(2,5-dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione**
Code name:	**BYI 08330-ketohydroxy**
Common name:	**Does not apply**
Empirical formula:	**$C_{18} H_{23} N O_4$**
Molar mass:	**317.39 g/mol**
Water solubility:	**Unknown**
n-Octanol/water partition coefficient:	**Unknown**
Labelling:	**azaspirodecenyl-3-^{14}C**
Specific radioactivity of the original radiolabelled batches:	**4.31 MBq/mg = 258,600 dpm/µg**
Specific radioactivity used for administration:	**4.31 MBq/mg = 258,600 dpm/µg**
Radiochemical purity:	**> 99 %**
Dose level:	**2 mg/kg body weight**
Vehicle:	**0.5 % aqueous tragacanth suspension**
Stability of the test material:	**The stability of [azaspirodecenyl-3-^{14}C]BYI 08330-ketohydroxy was demonstrated by radio HPLC analysis of the administration suspension immediately after dosing**

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2. Test Animals

Species:	**Rat (*Rattus norvegicus domesticus*)**
Strain:	**Wistar Hsd/Cpb: WU**
Breeding facility:	**[redacted] Germany**
Sex and numbers involved:	**Males: 4 animals**
Age:	**8 weeks at the time of delivery**
Body weight:	**Males: 200 – 212g at the time of administration 208 – 220 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 22 - 24 °C, relative humidity 50 – 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 [redacted], 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

The test item BYI 08330-ketohydroxy is a metabolite of BYI 08330 and was found in the target crop metabolism studies, where it was a main metabolite in cotton. Furthermore, BYI 08330-ketohydroxy was detected in the confined rotational crop study, where it was the main metabolite in Swiss chard and turnip roots and a main intermediate in the metabolic pathway. Based on these results, BYI 08330-ketohydroxy was included in the plant residue method and detected in fruiting and leafy crops of European residue trials (unpublished results).

BYI 08330-ketohydroxy was detected as a very minor metabolite only in the urine of the rat ADME study with the active ingredient BYI 08330. Therefore, a supplemental study was conducted to investigate the ADME behaviour of this compound in the rat.

Dosing

Each of the rats (4 animals per group) orally received 2 mg/kg bw of azaspirodecenyl-3-^{14}C labelled BYI 08330-ketohydroxy suspended in 0.5 % aqueous tragacanth after 16 hr fasting. The dosing

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suspension (0.2 mg /mL for the low and high dose level) was prepared one day before dosing and stored at 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). Actual administered doses were measured by LSC assay of aliquots of the administration suspensions. The actual mean administered dose of [azaspirodecenyl-3-^{14}C]BYI 8330-ketohydroxy was 1.94 mg/kg bw. The stability of the test compound in the suspension was determined by radio HPLC analysis of aliquots after administration.

Collection of blood
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 closed with adhesive tape. The capillaries were centrifuged at ca. 12000 g for about 10 minutes using a hematocrit centrifuge to separate the plasma from the formed constituents (mainly erythrocytes). After centrifugation, the capillary was broken at the border between plasma and formed constituents and the plasma (ca. 30 µL) was transferred onto a small metal dish for weighing. The dish was then placed into a scintillation vial for radioactivity measurement.

Collection of excreta
Urine was collected at various times separately for each animal in a cryogenic trap cooled with dry ice. 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. Urine samples were stored in a freezer before analysis.
Faeces were collected at various times separately for each animal in a cryogenic trap. The individual faeces samples were mixed with a small amount of water and stirred to prepare homogeneous slurry. The homogenised samples were weighed and the radioactivity of aliquots was determined by combustion/ LSC.

Sacrifice and organ/tissue sampling
At the respective test end (48 h after administration), the animals were anaesthetised using Pentobarbital-Na. They were sacrificed by transection of the cervical blood vessels. After transection of the cervical blood vessels, the oozed out blood was collected into test tubes coated with heparin and was separated afterwards into plasma and erythrocytes by centrifugation. The following organs/tissues were collected: erythrocytes, plasma, spleen, gastro-intestinal tract (GIT), liver, kidneys, perirenal fat, adrenal gland, testis, skeleton muscle, femur bone, heart, lung, brain, thyroid gland, skin, and carcass.
The organs and tissues prepared at the end the experiments were weighed immediately after the dissection and again following lyophilisation. Finally, they were homogenised and the radioactivity determined by combustion/LSC. The radioactivity remaining in the GIT, skin, and residual carcass was determined by combustion/LSC in order to establish the radioactivity balance. For the small organs and tissues (e.g. renal fat, adrenal glands, and thyroid), only the wet weight was determined and the samples then solubilised with BTS 450® (Beckman Tissue Solubiliser).

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Radioactivity measurement

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). For this purpose, the volume of the entire solution was determined and the radioactivity of an aliquot (1 – 3 replicates) of the solution was measured using the following scintillation counters:
Small organs or tissues were solubilised by means of a tissue solubiliser (e.g. BTS-450®). The solubilised samples or aliquots of them were acidified with hydrochloric acid or glacial acetic acid and mixed with a suitable scintillation cocktail (e.g. Quickszint 401, Zinsser Analytic GmbH). The radioactivity was then measured in a scintillation counter. This method was used for the following test materials: renal fat, adrenal glands, and thyroid.
Solid samples (1 – 5 aliquots) were weighed and combusted in an oxygen atmosphere using the following equipments Oxidizer 307/387 (Packard Instruments) for combustion of faeces, erythrocytes, lyophilised organs and tissues like e.g. spleen, liver, lung, bone muscle, gastrointestinal tract (GIT), residual carcass and solids (e.g. from faeces after extraction). Carbosorb E (9 mL) was used to trap the combustion product CO_2 and Permafluor E+ (10 mL; Packard Instruments) as scintillator for LSC.

Metabolite analysis

Urine samples were analysed without sample preparation. Faeces samples of all animals from 0 - 24 h post administration were combined and extracted successively 3 times with acetonitrile / water (8 : 2, v/v) using a Polytron homogeniser. Each extract was collected by centrifugation and its volume measured. Aliquots of each extract were radioassayed by LSC. The three extracts of each faeces preparation were combined and concentrated to the aqueous remainder and partitioned 3 times against n-hexane. Aliquots of aqueous and organic phases were radioassayed by LSC. The aqueous phase was concentrated and used for HPLC analysis. The radioactivity in post extraction solids was determined by combustion analysis.
The metabolites in the samples were quantified by HPLC using a reversed phase column (C18) and the eluting solvents water/formic acid = 99 : 1 (v/v) and acetonitrile/water/formic acid = 97 : 2 : 1 (v/v/v) in the gradient mode. Detection was performed by a UV (254 nm) and a radioisotope detector with a glass scintillator.
Metabolites in urine were identified by direct HPLC-MS-MS analysis of the urine pool 0 – 4 h. One major metabolite was also confirmed by HPLC co-chromatography with the non radiolabelled reference compound. Metabolites in faeces were mainly identified by comparison of the metabolic profile of faeces with urine. Two major metabolites were also identified by HPLC co-chromatography with reference compounds.

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II. Results and Discussion

A. Absorption

BYI 08330-ketohydroxy was rapidly absorbed from the gastrointestinal tract of male rats. The absorption commenced immediately after oral dosing. At least 55 % of the dose absorbed was derived from the rate of renal excretion and the residues in the body without gastro-intestinal tract at the time of sacrifice 48 h after dosing. The absorption rate was probably much higher since the metabolic profile in faeces was very similar to the one in urine, thus indicating the absorption and systemic availability of this part of the dose, too. Ca. 99 % of the administered radioactivity was recovered in urine, faeces, and the body of animals.

B. Distribution

Radioactivity in plasma of males and female rats following a single administration of 2 mg/kg bw reached its calculated maximum at 0.81 h postdose. Pharmacokinetic parameters were calculated from concentrations of total radioactivity using a 2 compartment disposition model. The calculated maximum radioactivity concentrations in plasma was 1.26 mg eq./kg bw. The peak concentration was slightly below the equal distribution concentration of 2 mg/kg bw BYI 08330-ketohydroxy.
Absorption was followed by a fast elimination phase. The mean residence time was low at 4.32 h.
Radioactivity levels in plasma are summarised in Table 5.1.1-19.
The distribution of radioactive residues in the body was analyzed at the time of sacrifice 48 h after dosing by radioactivity measurement of the major organs and tissues. Less than 0.2 % of the administered dose was detected in the body. Radioactivity concentrations were generally low with the highest levels detected in the gastro-intestinal tract, liver and kidney as the major organs responsible for metabolism and excretion. The distribution of radioactivity levels in organs and tissues of male and female rats after oral administration of [azaspirodecenyl-3-^{14}C]BYI 08330 is shown in Table 5.1.1-20.

C. Metabolism

The parent compound was not detected in urine. Traces of BYI 08330-ketohydroxy were found in faeces.
BYI 08330-ketohydroxy was completely metabolised forming numerous metabolites. The first and most important metabolic reaction was the oxidative demethylation of the cyclohexyl-O-methyl group to the respective alcohol (BYI 08330-desmethyl-ketohydroxy). Two isomers of BYI 08330-desmethyl-ketohydroxy were detected as the main components in excreta accounting for 15 % of the dose. All other identified metabolites could be derived from BYI 08330-desmethyl-ketohydroxy as intermediate. Most of the identified compounds were different mono-, di- and tri-oxygenated metabolites which are probably represented by isomers of mono-hydroxy, di-hydroxy and tri-hydroxy metabolites. A second oxidative transformation was formal loss of two hydrogen atoms of the oxygenated metabolites to “dehydro” derivates. These metabolites probably contain aldehyde or keto groups or carboxylic acid groups. LC-MS/MS clearly proved the metabolic transformations but did not allow to identify the positions of oxygenation and the functional groups formed.

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The oxygenated metabolites were classified in three groups to simplify evaluation and quantification: mono-hydroxy, di-hydroxy and tri-hydroxy metabolites of BYI 08330-desmethyl-ketohydroxy.

About 34 % of the dose was covered by the various different mono-hydroxy metabolites and 28 % by the group of di-hydroxy metabolites of BYI 08330-desmethyl-ketohydroxy. Tri-hydroxy metabolites were of minor importance. Only one metabolite of this group was detected accounting for about 3 % of the dose. Conjugation with e.g. glucuronic acid and sulfate was detected for few metabolites only and at low quantities. Cleavage of the azaspirodecane ring of BYI 08330-desmethyl-ketohydroxy was found to a low extent, which was proved by two metabolites: BYI 08330-desmethyl-MA-amide and BYI 08330-desmethyl-glyoxylic amide. Both metabolites covered $\leq$ 2.8 % of the dose. In total, 85.6 % of the administered dose were identified and 8.57 % were characterised. Only five minor metabolites all $<$1.6 % of the dose were not identified but characterised by their HPLC elution behaviour.

Metabolites excreted with urine and faeces following oral administration of [azaspirodecenyl-3-^{14}C] BYI 08330-ketohydroxy are shown in Table 5.1.1-22. The proposed metabolic pathway is presented in Figure 5.1.1-05.

D. Excretion

The excretion of BYI 08330-ketohydroxy was fast and almost completed 24 h after administration. About 54 % of the administered dose was eliminated via urine and about 44 % via faeces within 48 h after dosage. Quantitative recovery of radioactivity (98.75 % of dose) was accomplished.

The excretion of radioactivity following oral administration of [azaspirodecenyl-3-^{14}C] BYI 08330-ketohydroxy is shown in Table 5.1.1-21.

III. Conclusions

The fate of radiolabelled BYI 08330-ketohydroxy in male rats following a single low dose oral administration was characterized as follows:

- **Absorption**: Fast absorption of the radioactive test compound from the gastrointestinal tract with an absorption rate of at least 54 %
- **Distribution**: Fairly equal distribution of the radioactivity within the blood and most organs and tissues with preference to the liver as the main metabolizing organ also responsible for excretion
- Steady decline of radioactivity concentrations in plasma by several orders of magnitude within 48 hours
- Concentrations of radioactivity detected in tissues and organs at the time of sacrifice 48 hours postdose were very low
- **Excretion**: Rapid and complete excretion of radioactivity via the renal and faecal route
- **Metabolism**: Demethylation of the cyclohexyl methoxy group as the first metabolic transformation resulting in BYI 08330-desmethyl-ketohydroxy as the key intermediate in the metabolic pathway from which all other identified metabolites could be derived
- Oxidative conversion of BYI 08330-desmethyl-ketohydroxy to several isomers of mono-, di- and tri-oxygenated metabolites and corresponding "dehydro" metabolites

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These metabolites probably contain hydroxy groups, aldehyde or keto groups, and carboxylic acid groups at different positions in the molecule

- Cleavage of the azaspirodecane ring was detected as a minor metabolic reaction

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Table 5.1.1-19: Time course of radioactivity measured in the plasma of male rats after oral administration of 2 mg [azaspirodecenyl-3-^{14}C]BYI 08330-ketohydroxy/kg bw

Time [h p. admin.]	Equivalent Concentration [µg/g]
0.08	0.200
0.16	0.688
0.33	1.113
0.66	1.271
1.00	1.214
1.50	1.010
2.00	0.844
3.00	0.542
4.00	0.345
6.00	0.190
8.00	0.128
24.00	0.008
32.00	0.005
48.00	0.004

Table 5.1.1-20: Distribution of radioactivity in organs/tissues of male rats at sacrifice, 48 h after oral administration of 2 mg [azaspirodecenyl-3-^{14}C]BYI 08330-ketohydroxy/kg bw

Sample	Equivalent Concentration [µg/g]
Erythrocytes	0.0021
Plasma	0.0011
Spleen	0.0012
GIT	0.0103
Liver	0.0182
Kidney	0.0041
Perirenal fat	0.0009
Adrenal gland	0.0029
Testis	0.0036
Skel. Muscle	0.0024
Bone femur	0.0011
Heart	0.0012
Lung	0.0012
Brain	<LOD
Thyroid gland	0.0066
Skin	0.0020
Carcass	0.0026

<LOD value below detection limit

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Table 5.1.1-21: Time course of the cumulative excretion of radioactivity with urine and faeces of male rats after oral administration of 2 mg [azaspirodecane-3-^{14}C]BYI 08330-ketohydroxy/kg bw

Cumulative excretion of radioactivity in % of dose administered

Time [hours post admin.]	males 2 mg/kg bw
Urine	
4	23.83
8	43.16
12	48.53
24	53.74
48	54.45
Faeces	
24	41.45
48	44.09
Sum (48 h)	98.54

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Table 5.1.1-22: Distribution of parent compound and metabolites in the excreta of rats after oral administration of 2 mg [azaspirodecenyl-3-^{14}C]BYI 08330-ketohydroxy/kg bw

Time interval [h]			Percent of administered dose		
			urine 0 - 24 h	**faeces 0 - 24 h**	**Sum excreta**
% of dose analysed			53.75	40.46	94.20
Peak no.	Compound (report name) BYI 08330-	modification			
1	unknown		3.71	2.36	6.07
2	-desmethyl-ketohydroxy *	(+ O)	7.30	2.69	9.99
3	-desmethyl-ketohydroxy *	(+ O + Sulfate)	1.01	0.67	1.67
4	unknown		0.52	0.40	0.92
** 5			10.49	6.17	16.66
a	-desmethyl-ketohydroxy *	(+ 2x O - 2x H)			
b	-desmethyl-ketohydroxy *	(+ 2x O)			
c	-desmethyl-ketohydroxy *	(+ 2x O)			
d	-desmethyl-ketohydroxy *	(+ 2x O + conjugate)			
6	unknown		0.34	1.01	1.36
7	-desmethyl-ketohydroxy *	(+ O)	5.55	3.25	8.80
8	-desmethyl-ketohydroxy *	(+ O - 2x H)	3.01	0.08	3.09
9	-desmethyl-ketohydroxy *	(+ 3x O - 2x H)	1.44	1.63	3.07
10	-desmethyl-ketohydroxy *	(+ O)	4.36	0.62	4.98
11	-desmethyl-MA-amide		0.28	0.23	0.50
12	-desmethyl-ketohydroxy *	(+ O)	0.24	0.35	0.58
13	-desmethyl-ketohydroxy *	(+ 2x O - 2x H)	2.52	9.19	11.71
14	-desmethyl-ketohydroxy *	(+ O - 2x H)	0.60	---	0.60
15	-desmethyl-ketohydroxy *	(+ O)	0.31	0.29	0.60
16	unknown		0.14		0.14
17	-desmethyl-ketohydroxy *	(+ O)	0.50	0.19	0.69
18	-desmethyl-ketohydroxy	(Isomer of peak no. 23)	3.72	1.37	5.09
** 19			1.26	0.78	2.04
a	-desmethyl-ketohydroxy	(+ glucuronic acid)			
b	-desmethyl-ketohydroxy *	(+ O)			
** 20			1.42	0.85	2.27
a	-desmethyl-glyoxylic amide				
b	-desmethyl-ketohydroxy *	(+ 2x O - 2x H)			
21	-desmethyl-ketohydroxy *	(+ O)	0.95	1.00	1.95
22	-desmethyl-ketohydroxy *	(+ O)	0.54	0.28	0.82
23	-desmethyl-ketohydroxy		3.46	6.15	9.60
24	unknown		0.09	---	0.09
25	-ketohydroxy		---	0.90	0.90
Identified:			**48.95**	**36.68**	**85.63**
Characterised			4.80	3.78	8.57
Total analysed:			**53.75**	**40.46**	**94.20**
Not analysed (urine + faeces 24 - 48 h)			0.71	2.64	3.34
Solids (from faeces extraction)				0.96	0.96
Organic phase (from faeces extraction)				0.03	0.03
Total in excreta			**54.45**	**44.09**	**98.54**

* = derivate of BYI 08330-desmethyl-ketohydroxy

** = peak 5 consists of four components, and the peaks 19 to 20 each of two components

-- not detected

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Figure 5.1.1-05 Proposed metabolic pathway of BYI 08330-ketohydroxy in the rat

BYI 08330-ketohydroxy

-desmethyl-ketohydroxy

conjugation with glucuronic acid

conjugation with sulfuric acid

-desmethyl-glyoxylic -amide

-desmethyl-MA-amide

+ O

+ 2 O

+ 3 O

+ O - 2 H

+ 2 O - 2 H

+ 3 O - 2 H

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IIA 5.1.2 Toxicokinetic studies - Second single dose, oral route, in rats

BYI 08330 was not degraded to $^{14}CO_2$ and volatile metabolites in the single dose autoradiography study with the azaspirodecenyl-3-^{14}C label (Report No. MEF06/15). No hints for molecular cleavage were detected and the azaspirodecenyl moiety was conserved in all metabolites identified in the ADME study (Report No. MEF-048/04). The identification rates were high in the range of 87 – 94 % of the administered dose.

Therefore, a study with a second radiolabel was not considered necessary.

IIA 5.1.3 Toxicokinetic studies - Repeated dose, oral route, in rats

Two repeated dose tests with male and female rats were conducted within the ADME study (Report No. MEF-048/04). Male and female rats received 14 daily oral doses of 2 mg/kg bw non-radiolabelled BYI 08330 followed by a single oral dose of 2 mg/kg bw [azaspirodecenyl-3-^{14}C]BYI 08330. All the results of these tests were very similar to those of the single oral dose tests in males and females. No accumulation, retention or delayed excretion was detected compared to the single low dose tests.

All details are presented and discussed in IIA 5.1.1 (study 5.1.1/02).

IIA 5.1.4 Special in vitro studies – Transporter Assays

Report:	KIIA 5.1.4/01, [redacted] 2011
Title:	Inhibitory Potential of BYI08330-enol and BYI08330-desmethylenol as inhibitors on hOAT1, hOAT3 and hOAT4 in transfected HEK-cells
Report No & Document No	PCT-009-11 M-413065-01-1
Guidelines:	Not applicable
GLP	No
Testing Facility and Dates	[redacted] Germany Experimental work: APR 29, 2011 – MAY 30, 2011

Executive Summary

Based on pharmakokinetik data and pharmakokinetik modelling it is assumed that the BYI08330-enol and the BYI08330-desmethylenol will be distributed and eliminated (mainly via urine) by membrane transporters. Both metabolites are weak acids and thus present as monovalent anions at physiological pH, and therefore cannot diffuse efficiently through cell membranes.
At spirotetramat doses above 300 mg/kg bw/day, active transport capacity seems to become saturated leading to an accumulation of these main metabolites in the body. This is also suggested by a mechanistic

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study in rats (, 2005, M-252001-01-2). More than 10 daily high doses of spirotetramat were necessary to elicit sperm cell effects in rats.

In order to investigate whether BYI08330-enol ("E") and BYI08330-desmethylenol ("DME") are substrates or inhibitors of human organic anion transporters (hOAT), transporter assays using hOAT-transfected human embryonic kidney (HEK)-cell lines were performed. The aim of the study is to characterize whether E and DME are interacting with hOAT1-mediated p-aminohippuric acid (PAH) uptake and with hOAT3- as well as hOAT4-mediated estrone sulfate (ES) uptake, respectively.

In the ADME study in rats (, 2006; M-268709-02-2), maximum plasma concentrations (C_{max}) of approximately 10 to 550 µM of E and DME have been reached in male rats after a single low and moderate dose of spirotetramat (i.e. 2 and 100 mg/kg bw). Therefore, 20 µM and 200 µM of the enol and the desmethyl-enol were used in these OAT-interaction studies

Two concentrations of PAH and ES at the Km and 1/10 Km value, determined by PortaCellTec biosciences, were used to characterize the inhibitory potential of E and DME. Probenecid, a well known competitive inhibitor of hOAT1 and hOAT3, and Sulfobromophthalein disodium salt hydrate (BSP), an inhibitor of hOAT4, were used in the experiments as positive controls.

The results of the studies can be summarized as follows:

hOAT1-mediated [^{3}H]PAH uptake was significantly inhibited by 200 µM E by a maximum value of 36±2% (100 µM PAH). The second test substance DME showed no interaction with the OAT1-mediated PAH uptake.

hOAT3-mediated [^{3}H]ES uptake was significant inhibited by both concentrations of E by a maximum value of 34±2% (20 µM) and 76±1% (200 µM). The test substance DME had a much lower inhibitory effect on the OAT3-mediated ES-uptake (1 µM) with values up to a maximum 10±8% (20 µM) and 24±4% (200µM).

hOAT4- mediated [^{3}H]ES uptake was significantly reduced by both test substances. The inhibitory effect was concentration dependent with a maximum value at the lower ES concentration (1 µM) of 73±1 (200 µM E) and 62±0.3% (200 µM DME), respectively.

In conclusion, BYI08330-enol and BYI08330-desmethylenol clearly interact with the human OAT transporters. Membrane transporter proteins involved in the renal elimination of xenobiotics may become saturated after exaggerated high experimental doses. This may lead to a transition from first order kinetics to zero-order elimination kinetics. Thus, identification of transporter interactions may help in predicting the pharmacokinetics of drugs and explaining its toxic effects at high dose levels.

Since substrates affinity and inhibitory potential of human, mouse and rat OATs are highly overlapping, it is inferred that the BYI08330-enol and the BYI08330-desmethylenol are also eliminated via rat organic anion transporter orthologs in the kidneys.

Because the enol metabolite of spirotetramat was found to be partly conjugated by glucuronic acid in mice and humans (, 2006; M-274118-01-2), other important transporters like OATPs, mainly expressed in the liver, and also the efflux multidrug transporters (e.g. MDR1, MRP2, MRP4) could also be involved in the elimination of this conjugated metabolite.

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I. Material and methods

A. Materials:

1. Test Material:	BYI08330-enol (“E”) and BYI08330-desmethylenol (“DME”)
Description:	beige powder (E)
	white solid (DME)
Lot/Batch No.:	692-101-09-0004 (E)
	FHN 20335-2-2 (DME)
Purity:	99.1% (MAR 11, 2011) (E)
	96.1 % (FEB 26, 2011) (DME)
Molar Mass:	301 g/mol (E)
	287 g/mol (DME)
Chemical Name:	3-(2,5-dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one (E)
	(5s, 8s)-3-(2,5-dimethylphenyl)-4,8-dihydroxy-1-azaspiro[4.5]dec-3-en-2-one (DME)
Concentration tested	20 µM and 200 µM (for both E and DME)
Vehicle	2 mM stock solution in experimental buffer (HBSS, supplemented with 20 mM HEPES, pH 7.4)
2.Substrates	
for hOAT1:	p-Aminohippuric acid (PAH) ███-Aldrich (Lot no. 0001433124)
	$[^{3}H]$-p-Aminohippuric acid (aminohippuric acid, P-[GLYCYL-2-^{3}H]) PerkinElmer (Lot no 3632431)
for hOAT3+4:	Estrone 3-sulfate (ES)
	$[^{3}H]$Estrone sulfate, ammonium salt, [6,7-^{3}H(N)] PerkinElmer (Lot no 3632437)
3.Inhibitors	
for OAT 1+3:	Probenecid (*p*-(Dipropylsulfamoyl) benzoic acid) ███-Aldrich (Lot no. 118K0893)
for OAT 4:	Sulfobromophthalein disodium salt hydrate (BSP) Carl Roth GmbH&Co KG (Lot no. 499150456)
4. Test System	Human Embryonic Kidney Cells (HEK) hOAT transfected and vector-transfected, cultured for 3 days
Density:	2 x 10^{5} cells / well

B. Study design:

Preparation of Test Item Solutions

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A 2 mM stock solution in experimental buffer (HBSS, supplemented with 20 mM HEPES, pH 7.4) was prepared for unlabeled E and DME. Each solution was aliquoted and stored at 4°C. Additional working solutions of E and DME were freshly prepared as necessary in experimental buffer (HBSS, supplemented with 20 mM HEPES, pH 7.4).

<u>Preparation of Stock and Working Solutions</u>

The stock solutions of ES (MW: 388.52 g/mol) and BSP (MW: 838 g/mol) were prepared in water in a final concentration of 50 mM. The stock solution of PAH (MW: 194.19 g/mol) was prepared in water in a final concentration of 100 mM. Each solution was aliquoted and stored at -20°C. Additional working solutions of ES, BSP, and PAH were freshly prepared as necessary in water.
The stock solution of probenecid (MW: 285.36 g/mol) was prepared in dimethyl sulfoxide (DMSO for molecular biology) in a final concentration of 100 mM. Additional working solutions of probenecid were freshly prepared as necessary in DMSO.
The final incubation solutions were prepared in HBSS (supplemented with 20 mM HEPES, pH 7.4) by serial dilutions such that the DMSO content was equal to 1% (v/v). All vector-transfected control cells were also treated with 1% DMSO.
The $[^3H]$PAH and $[^3H]$ES working solutions were prepared in experimental buffer.

<u>Thawing Procedure and Proliferation Conditions</u>

All cells were handled under a laminar flow. For disinfection purpose, all critical instruments for handling of cells were purified with 70% ethanol in advance.
Cryogenic vials containing about 3×10^6 hOAT-transfected or vector-transfected HEK cells were thawed in a water bath at 37°C until a small rest of ice was present. The cell suspension was immediately and completely transferred into a cell culture dish 100 mm containing 10 mL of 37°C-tempered PAA-medium (PAA, Quantum 286 for epithelial cells with L-glutamine, 1% penicillin/streptomycin). The hOAT3-transfected cells were cultured in PAA-medium additionally containing 175 µg/mL hygromycine B. After 24 h the medium was replaced by new medium.
OAT-transfected and vector-transfected HEK cells were grown on 100 mm or 150 mm cell culture dishes at 37°C in a humidified 5% CO_2 atmosphere.
All HEK cell lines (hOAT1, hOAT3, hOAT4 and vector-HEK-cells) were tested for mycoplasma - before starting transport experiments using Mycoplasma Detection Kit for conventional PCR

<u>Passaging of HEK Cell Lines</u>

After removal of the medium, the cells were washed with 5-10 mL of PBS. The adherent cells were exposed to 1-2 mL trypsin-EDTA solution for 5 min at room temperature. To the cell culture plate 3-4 mL medium was added and the suspension was transferred into a falcon tube and centrifuged at 124 x g for 3 min. The cell pellet was resuspended in about 1-3 mL medium.
Confluent grown cell cultures were split twice a week 1:4 to 1:10. For counting in a Neubauer-counting chamber, cells were diluted 1:20 in medium. A fraction of harvested cells was used for transport experiments.

<u>Preparation of HEK Cells for Uptake Assays</u>

For uptake assays, 24-well plates were pretreated with poly-D-lysine hydrobromide solution (2 mg/mL). Therefore, each well was coated with 0.5 mL poly-D-lysine solution and incubated for at

least 15-30 min. After complete removal of the solution the plates were dried for 30-60 min. Before use in transport experiments freshly harvested cells were seeded into 24 well plates (2 x 10^5 cells in 0.5 mL medium per well) and cultured for 3 days

OAT Uptake Assay

Growth medium was aspirated and each well was rinsed twice with 0.5 mL PBS buffer and then preincubated in 0.5 mL incubation buffer HBSS (supplemented with 20 mM HEPES, pH 7.4) for 20 min at 37°C. The incubation buffer was removed and 200 µL incubation buffer containing the radiolabeled and non-radiolabeled substances was added to each well and incubated at 37°C for designated time intervals. After incubation, the uptake was terminated by aspirating the reaction mixture and washing the cells three times with 1 mL ice-cold PBS buffer. Cells were solubilized with 0.5 mL of 1N NaOH over night. [^{3}H] content was measured after addition of 2.5 mL scintillation solvent in a LS 6000 Beckmann scintillation counter.

Uptake Transport Experiments in hOAT1-transfected HEK Cells

The PAH incubation buffer contained [^{3}H]PAH and unlabeled PAH in a final concentration of 10 µM and 100 µM, respectively. To characterize the inhibitory potential of “E” and “DME” each test item was added in two different concentrations to 10 and 100 µM [^{3}H]PAH, respectively. Inhibition of hOAT1-mediated PAH uptake by 100 µM probenecid was performed as control experiment. In detail the following concentrations were used:

Substrate	I. Substrate Concentration (µM)	II. Substrate Concentration (µM)	Inhibitor	Inhibitor Concentration (µM)
PAH	10	100	-	-
	10	100	E	20
	10	100	DME	200
	10	100	Probenecid	100

After 3 min the incubation was terminated. All experiments were conducted at least on 2 separate days. On each day, all experiments were performed as triplicates.

Uptake Transport Experiments in hOAT3-transfected HEK Cells

The ES incubation buffer contained [^{3}H]ES and unlabeled ES in a final concentration of 1 µM and 10 µM, respectively. To characterize the inhibitory potential of “E” and “DME” each test item was added in two different concentrations to 1µM and 10 µM [^{3}H]ES, respectively. Inhibition of hOAT3-mediated ES uptake by 100 µM probenecid was performed as control experiment. In detail the following concentrations were used:

Substrate	I. Substrate Concentration (µM)	II. Substrate Concentration (µM)	Inhibitor	Inhibitor Concentration (µM)
ES	1	10	-	-
	1	10	E	20
	1	10	DME	200

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	1	10	Probenecid	100

The incubation was terminated after 1 min. All experiments were conducted at least on 2 separate days. On each day, all experiments were performed as triplicates.

Uptake Transport Experiments in hOAT4-transfected HEK Cells
The ES incubation buffer contained [^{3}H]ES and unlabeled ES in a final concentration of 1 µM and 10 µM, respectively. To characterize the inhibitory potential of "E" and "DME" each test item was added in two different concentrations to 1 µM and 10 µM [^{3}H]ES, respectively. Inhibition of hOAT4-mediated ES uptake by 10 µM BSP was performed as control experiment. In detail the following concentrations were used:

Substrate	**I. Substrate Concentration (µM)**	**II. Substrate Concentration (µM)**	**Inhibitor**	**Inhibitor Concentration (µM)**
ES	1	10	-	-
	1	10	E	20
	1	10	DME	200
	1	10	BSP	10

The incubation was terminated after 1 min. All experiments were conducted at least on 2 separate days. On each day, all experiments were performed as triplicates.

Determination of the Protein Amount
Cellular protein amount was determined in parallel from 6 wells from a different 24-well reference plate per cell line and experimental day using a method described by Bradford 1976 [8].

For 250 mL Bradford-reagent (4x) were used: 70 mg Serva Blue G
50 mL ethanol (96%)
100 mL phosphoric acid (85%)

Cell monolayers in 24-well plates were washed 3 x with 500 µL PBS buffer and incubated for lysis 30-60 min in 100 µL 1 x lysis buffer (5 x lysis buffer; Promega; diluted 1:5 in PBS buffer) per well. Cell lysate was filled up with PBS buffer to 1 mL per well and mixed vigorously. Plates were stored at -20°C.
Protein determination was performed in 96 well plates (Sarstedt; flat bottom) in duplicate. BSA (stock solution: 1 mg/mL) was used as standard for a calibration curve with the following concentrations (0, 50, 75, 100, 150, 200, 250, 300 µg/mL)
20 µL of BSA-standards or 20 µL sample was mixed with 200 µL Bradford-reagent (diluted 1:4 in PBS buffer from stock reagent) per well. After 10-20 min incubation at room temperature, absorption was measured at 590 nm (Microplate Reader, Mithras LB 940, Berthold).

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Data Analysis and Results

The absolute amount (pmol) of the substrate uptake was calculated within the given time and related to the determined protein values. Initially, the specific activity was calculated according the labeling (µCi/mmol) and the concentration of the substrate. Additionally, the radioactivity (dpm) of aliquots of each substrate solution was determined. From this, the specific radioactivity, so called "*standard*", was calculated according the following formula:

$$SA = \frac{St_{dpm}}{[S] * V_s * 10^6}$$

SA: specific radioactivity (dpm/pmol)
St_{dpm}: mean radioactivity of standards (dpm)
[S]: substrate concentration (µmol/L)
V_s : volume of the aliquot substrate solution (L)

The specific radioactivity from each condition was used to determine the uptake values U of every sample, which was calculated according the following formula:

$$U = \frac{RA_{sample}}{SA * P}$$

U: uptake (pmol/mg protein)
SA: specific radioactivity (dpm/pmol)
RA_{sample}: radioactivity of one well of the 24 well plate (dpm)
P: protein amount (mean of six wells from the 24 well reference plate) (mg protein)
Initial uptake rate v for each well was calculated according to the following formula:

$$v = \frac{U}{t}$$

v: uptake rate (pmol/mg protein/min)
U: uptake (pmol/mg protein)
t: incubation interval (min)

OAT-transporter mediated uptake rate (net-uptake) was obtained by subtracting the uptake rate in vector-transfected HEK cells from the uptake rate in OAT-HEK cells as described below:

$$v^{OAT} = v^{OAT-HEK} - v^{vector-HEK}$$

v^{OAT}: initial uptake rate (OAT mediated) (pmol/mg protein/min)
v: initial uptake rate (pmol/mg protein/min)

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The percentage of uptake inhibition was calculated from the net-uptake from control experiments in the absence of added inhibitor (100%). Means ± average deviation (av. dev.) were calculated from two independent experiments on 2 separate days.

II. Results and discussion:

Inhibition of hOAT1 mediated [^{3}H]PAH uptake by E and DME

To determine, if E and DME are hOAT1 inhibitors, the inhibitory potential towards the uptake of a probe substrate was analyzed.

For these inhibition studies PAH was used as a specific hOAT1 probe substrate at concentrations of 10 µM and 100 µM [9, 10]. The inhibitory effect of E and DME on the hOAT1 mediated [^{3}H]PAH uptake was evaluated at two concentrations (20 and 200 µM).

The test item E shows only at a higher concentration of 200 µM a significant inhibition of the hOAT1-mediated [^{3}H]PAH uptake. The uptake of [^{3}H]PAH was reduced by 35% (10 µM PAH) and by 36% (100 µM). Two tested concentrations of DME (20 µM and 200 µM) had no inhibitory effect on the [^{3}H]PAH net-uptake (10 µM and 100 µM) in hOAT1-transfected HEK cells. Probenecid was used as a specific positive control inhibitor on the hOAT1 mediated [^{3}H]PAH uptake. In these two experiments 100 µM of probenecid showed a significant inhibition of 10 µM [^{3}H]PAH (89%) and of 100 µM [^{3}H]PAH (82%), respectively.

Inhibition of hOAT3 mediated [^{3}H]ES uptake by E and DME

To determine, if E and DME are hOAT3 inhibitors, the inhibitory potential towards the uptake of a probe substrate was analyzed.

For inhibition studies [^{3}H]ES was used as a specific hOAT3 substrate at concentrations of 1 µM and 10 µM [9, 10]. The inhibitory effect of E and DME towards the hOAT3 mediated [^{3}H]ES uptake was evaluated at two concentrations (20 µM and 200 µM).

The test item E showed a significant concentration dependent inhibition of the hOAT3-mediated ES uptake. The inhibitory potential of E on the ES uptake (1 µM) was 34±1.8% (20 µM) and 76±0.5 % (200 µM), respectively. At a higher ES concentration of 10 µM ES the inhibitory effect of E was lower with 16±1.9% (20 µM) and 59±3 % (200 µM), respectively. DME showed a slight inhibition of the [^{3}H]ES net-uptake in hOAT3-transfected HEK cells. At a tested concentration of 1µM [^{3}H]ES the inhibitory effect of 20 µM and 200 µM was 10±7.5% and 24±4%, respectively. Probenecid was used as a specific positive control inhibitor on the hOAT3 mediated [^{3}H]ES uptake. In this experiments 100 µM of probenecid showed a significant inhibition of 1 µM [^{3}H]ES (94%) and 10 µM [^{3}H]ES (87%), respectively.

Inhibition of hOAT4 mediated [^{3}H]ES uptake by E and DME

To determine, if E and DME are hOAT4 inhibitors, the inhibitory potential towards the uptake of a probe substrate was analyzed.

For inhibition studies [^{3}H]ES was used as a specific hOAT3 substrate at concentrations of 1 µM and 10 µM [9, 10]. The inhibitory effect of E and DME towards the hOAT4 mediated [^{3}H]ES uptake was evaluated at two concentrations (20 µM and 200 µM).

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The test item E showed a significant concentration dependent inhibition of the hOAT4-mediated ES uptake. The inhibitory potential of E on the ES uptake (1 µM) was 27±2.0% (20 µM) and 73±1.0% (200 µM), respectively. At a higher ES concentration of 10 µM the inhibitory effect of E was slightly reduced with 15±0.4% (20 µM) and 59±2.0% (200 µM), respectively. DME showed also a concentration dependent inhibition of the [^{3}H]ES net-uptake in hOAT3-transfected HEK cells. With the concentration of 1µM [^{3}H]ES the inhibitory effect of the highest tested concentration of DME (200 µM) was 62±0.2%. With the concentration of 10µM [^{3}H]ES the inhibitory effect of the highest tested DME concentration (200 µM) was slightly lower with 52±3%. BSP was used as a specific positive control inhibitor on the hOAT4 mediated [^{3}H]ES uptake. In this experiments 10 µM of BSP showed a significant inhibition of 1 µM [^{3}H]ES (95%) and 10 µM [^{3}H]ES (92%), respectively.

III. Conclusion:

The human OAT transporters interact with the BYI08330-enol and/ or the BYI08330-desmethylenol. Membrane transporter proteins involved in the renal elimination of xenobiotics may become saturated after exaggerated high experimental doses. This may lead to a transition from first order kinetics to zero-order kinetics, implying that the elimination rate is no longer proportional to the drug concentration. Thus, identification of transporter interactions may help in predicting the pharmacokinetics of drugs and explaining its toxic effects at high dose levels.

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IIA 5.2 Acute toxicity

Summary of acute toxicity studies

Type of study	**Vehicle**	**LD_{50}/LC_{50} / result**	**Report No.**
Acute oral, rat	carboxymethyl-cellulose sodium salt solution	>2,000 mg/kg bw LD50 oral cut-off > 5,000 mg/kg (according to OECD 423; Category 5 / unclassified based on the Globally Harmonized Classification System)	200398
Acute neurotoxicity, rat	0.5% methylcellulose /0.4% Tween 80 in deionized water	NOAEL = 100 mg/kg based on urine stains in both genders and decreased motor activity in male rats at 200 mg/kg (no evidence of a neurotoxic potential)	201283
Acute dermal, rat	carboxymethyl-cellulose sodium salt solution	>2,000 mg/kg bw	200399
Acute inhalation, rat (solid aerosol)	none	> 4,183 mg/m^3 air	32020
Skin irritation, rabbit	water	non-irritant	R 8147
Eye irritation, rabbit	-	irritant	R 8146
Skin sensitization, guinea pig (Maximization Test)	propylene glycol 400	skin sensitizing potential	32273
Skin sensitization, guinea pig (Buehler Test)	propylene glycol 400	no evidence of skin sensitization	AT01317
Skin sensitization, mice (LLNA)	dimethylformamide	skin sensitizing potential	SA04120

BYI 08330 has a very low acute oral (LD50 oral > 5,000 mg/kg), dermal (LD_{50}: >2,000 mg/kg bw) and inhalative toxicity (LC_{50}: > 4,183 mg/m^3 air; the mean maximum attainable concentration) in male and female rats. BYI 08330 is non-irritating to the skin but irritating to the eyes. BYI 08330 exhibits a skin sensitization potential under the conditions of the guinea pig maximization test and the Local Lymph Node Assay. A Buehler Patch Test was negative. Based on these results, BYI 08330 will be in EPA Category II for all routes of acute exposure.

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IIA 5.2.1 Acute oral toxicity

Report:	**KIIA 5.2.1/01, ; 2004**
Title:	An Acute Oral LD_{50} Study in the Rat with BYI 08330
Report No & Document No	200398 M-069299-01-1
Guidelines:	US-EPA-OPPTS, 870.1100; OECD Guideline No. 425. Deviation(s): none
OECD/FIFRA GLPS	Yes. Deviation(s): none

Executive Summary

In an acute oral toxicity study, one group of five fasted, young adult female Wistar Hanover (Crl:WI[Glx/BRL/Han]IGS BR) rats were given a single oral dose (gavage) of 2,000 mg/kg of BYI 08330 (batch no. NLL6425-9, purity: 93.5%) suspended in 0.5% aqueous carboxymethylcellulose and were observed for 14 days.
BYI 08330 was found to have virtually no acute oral toxicity following exposure of rats. Clinical signs were not observed during the course of the study and there were no effects on body weight development. On the basis of this study, BYI 08330 does not warrant classification as being harmful or toxic.

I. Material and methods

A. Materials:

1. Test Material:	BYI 08330
Description:	Technical grade, beige powder
Lot/Batch No.:	NLL 6425-9
Purity:	93.5 % (6/21/02), 93.1 % (1/15/03)
Compound Stability:	Stable at room temperature (~22 °C)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1
2. Vehicle and/or positive control:	BYI 08330 was formulated, the day of dosing, as a suspension in 0.5% aqueous carboxymethylcellulose
3. Test animals:	
Species:	Rat, females
Strain:	Wistar Hanover (Crl:WI[Glx/BRL/Han]IGS BR)
Age/weight at study initiation:	10 weeks of age / Initial body weight range for all five females was 155 g – 159 g
Source:	

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Acclimatisation:	September 23, 2002 to October 4, 2002
Housing:	Individually housed in stainless steel wire mesh cages providing a 66.5 square inch area. Minimum space requirement for rats weighing less than 500 grams is 60 square inches
Diet:	Rodent Chow No.5002, *ad libitum*
Water:	Kansas City Water District provides water for our facility. Water is provided *ad libitum* using an Edstrom automatic watering system
Environmental conditions:	**Temperature and Humidity:** Temperature: Set to be maintained at: 19° to 25°C (66° to 77°F); Humidity: Set to be maintained at: 30% to 70% RH
	Lighting: 12-hour light/dark cycle
	Air changes: Airflow in the animal study room, calculated as air changes per hour, averaged at least twelve changes per hour from September 23, 2002, until October 30, 2002. Five instances of less than ten changes per hour were recorded, but the low reading did not cause the daily average to fall below twelve changes per hour

B. Study design and methods:

1. In life dates: October 22, 2002 – November 6, 2002

2. Animal assignment and treatment:

The oral route of exposure was employed in accordance with the test guidelines for an acute oral toxicity study. The BYI 08330 was formulated, the day of dosing, as a suspension in 0.5% aqueous carboxymethylcellulose. (Note: The BYI 08330 had to be mixed for approximately two hours to form a suspension which could be drawn through the gavage needle.) The BYI 08330 was administered via gavage, at a dose volume of 10 ml/kg, to rats which had been fasted overnight. The dose used in this study was 2,000 mg/kg.

At the day of dosing, the dosed animals were observed for detailed clinical observations: 1) within 30 minutes of dosing, 2) two observations were made between 30 minutes and four hours after dosing, and 3) in the afternoon on the day of dosing. Following the day of dosing, clinical observations were made once each morning and a mortality check was performed each afternoon, except on weekends. Body weights were recorded on the day of dosing, day 7, and day 14 (terminal body weight). Surviving animals were sacrificed by CO_2 asphyxiation 14 days after having been dosed with BYI 08330. All animals were subjected to a complete postmortem (gross) examination.

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II. Results and discussion:

Mortality:

No mortalities were observed.

Table 5.2.1-1 Doses, mortality / animals treated

Dose [mg/kg bw]	Toxicological results#			Duration of signs	Time of death	Mortality [%]
Females						
2,000	0	0	5	-	-	0
LD$_{50}$ (females): > 2,000 mg/kg bw						

1st figure = number of dead animals; 2nd figure = number of animals with signs; 3rd figure = number of animals in the group

The acute oral LD$_{50}$ of BYI 08330 was > 2,000 mg/kg bw. According to OECD guideline 423, the LD$_{50}$ can be classified as LD50 oral > 5,000 mg/kg (Category 5 / unclassified based on the Globally Harmonized Classification System).

Body weight:

There were no treatment-related effects on body weights or body weight gain.

Clinical signs:

No treatment-related findings.

Necropsy:

No treatment-related findings.

III. Conclusion: BYI 08330 has virtually no acute oral toxicity in rats.

Classification/labelling according to Commission Directive 67/548/EEC: none

EPA Category IV

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IIA 5.2.2 Acute percutaneous toxicity

Report:	**KIIA 5.2.2/01, [redacted] 2004**
Title:	An Acute Dermal LD_{50} Study in the Rat with BYI 08330
Report No & Document No	200399 M-066937-01-2
Guidelines:	US-EPA-OPPTS, 870.1200; OECD Guideline No. 402, JMAFF Guideline No. 8268. Deviation(s): none
OECD/FIFRA GLPS	Yes. Deviation(s): none

Executive Summary

In an acute dermal toxicity study, groups of young adult Wistar Hanover (Crl:WI[Glx/BRL/Han]IGS BR) rats (5/sex/dose) were exposed by the dermal route to BYI 08330 (batch no. NLL6425-9, purity: 93.5%). The test material was mixed with aqueous 0.5% carboxymethylcellulose in approximately a 1:1 ratio (weight / weight), to form a dry paste and applied at a dose of 2,000 mg/kg bw for 24 hours under occlusive conditions to the shaved dorsal skin of each animals body surface (25 cm^2).

BYI 08330 was found to have a low acute dermal toxicity following exposure of rats. Clinical signs included red stains at the nose, wetness and yellow stains in the urogenital area, and a red zone in the back. There were no effects on body weight development. On the basis of this study, BYI 08330 does not warrant classification as being harmful or toxic.

I. Material and methods

A. Materials:

1. Test Material:	BYI 08330
Description:	Technical grade, beige powder
Lot/Batch No.:	NLL 6425-9
Purity:	93.5 % (6/21/02), 93.1 % (1/15/03)
Compound Stability:	Stable at room temperature (~ 22 ºC)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1
2. Vehicle and/or positive control:	BYI 08330 was mixed with aqueous 0.5% carboxymethylcellulose in approximately a 1:1 ratio (weight to weight), to form a dry paste
3. Test animals:	
Species:	Rat, males and females
Strain:	Wistar Hanover (Crl:WI[Glx/BRL/Han]IGS BR)

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Age/weight at study initiation:	10 weeks of age / initial body weight range for all animals that were dosed with BYI 08330 was 182 g - 195 g for females and 271 g – 319 g for males	
Source:	███████████	
Acclimatisation:	September 23, 2002 to October 4, 2002	
Housing:	Individually housed in stainless steel wire mesh cages providing a 66.5 square inch area. Minimum space requirement for rats weighing less than 500 grams is 60 square inches	
Diet:	███████ Rodent Chow No.5002, *ad libitum*	
Water:	Kansas City Water District provides water for our facility. Tap water is provided *ad libitum* using an Edstrom automatic watering system.	
Environmental conditions:	**Temperature and Humidity:**	Temperature: Set to be maintained at:19° to 25°C (66° to 77°F); Humidity: Set to be maintained at: 30% to 70% RH
	Lighting:	12-hour light/dark cycle
	Air changes:	Airflow in the animal study room, calculated as air changes per hour, averaged at least twelve changes per hour from September 23, 2002, until October 30, 2002. Five instances of less than ten changes per hour were recorded, but the low reading did not cause the daily average to fall below twelve changes per hour.

B. Study design and methods:

1. In life dates: October 22, 2002 – November 5, 2002

2. Animal assignment and treatment:

The BYI 08330 was mixed with aqueous 0.5% carboxymethylcellulose in approximately a 1:1 ratio (weight / weight), to form a dry paste. The paste was placed on a 2 inch by 2 inch piece of 2 ply gauze, which was backed with a piece of plastic, and the gauze was applied to the shaved area of the back. The gauze was secured with porous medical tape and the torso of the animals was wrapped with porous medical tape (Zonas®, by Johnson and Johnson). The BYI 08330 was held in contact with the skin for a minimum of 24 hours. After this, the wrappings were removed and the dose site was gently wiped with water-dampened and dry paper towels to remove as much test substance residue as feasible without damaging the skin. Clinical observations were made once each morning, except on the first two days of the study when clinical observations were also performed in the afternoon. A mortality check was performed each afternoon, except on weekends. Body weights were recorded on the day of dosing, day 7, and day 14 (terminal body weight). Surviving animals were sacrificed by CO_2 asphyxiation 14 days after having been dosed with BYI 08830. All animals were subjected to a complete postmortem (gross) examination.

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II. Results and discussion:

Mortality:
No mortalities were observed.

Table 5.2.2-1 Doses, mortality / animals treated

Dose [mg/kg bw]	Toxicological results#			Duration of signs	Time of death	Mortality [%]
Males						
2,000	0	2	5	1 – 3 days	-	0
LD_{50} (males): >2,000 mg/kg bw						
Females						
2,000	0	5	5	1 – 3 days	-	0
LD_{50} (females): >2,000 mg/kg bw						

1st figure = number of dead animals; 2nd figure = number of animals with signs; 3rd figure = number of animals in the group

Body weight:
No treatment-related findings
Clinical signs:
Clinical signs observed on days 0-3 were: nose: red stain, urogenital area: wetness, urogenital area: yellow stain, back: red zone.
Necropsy:
No treatment-related findings.

III. Conclusion: BYI 08330 has a very low acute dermal toxicity in rats.
Classification/labelling according to Commission Directive 67/548/EEC: none
EPA Category III

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IIA 5.2.3 Acute inhalation toxicity

Report:	**KIIA 5.2.3/01, [redacted] 2002**
Title:	Study on acute inhalation toxicity in rats according to OECD No. 403
Report No &	32020
Document No	M-064654-01-2
Guidelines:	OECD Guideline No. 403; 92/69/EEC; US-EPA OPPTS 870.1300. Deviation(s): none
OECD/FIFRA GLPS	Yes. Deviation(s): none

Executive Summary

In an acute inhalative toxicity study, two groups of Wistar (HsdCpb:WU (SPF-bred)) rats (5/sex/group) were nose-only exposed to mean solid aerosol concentrations of 1,100 and 4,183 mg BYI 08330/m³ air, the maximum technically attainable gravimetric concentration (batch no. NLL6425-9, purity: 96.5%). The aerosol generated was respirable to rats. The results can be summarized as follows:

LC50 inhalation (solid-aerosol, 4 hr): > 4,183 mg/m³)

NOAEL Males & females: < 1,100 mg/m³ air

Exposure up to 4,183 mg/m³ did not result in mortality. The following clinical signs were noted within the observation period of 14 days: ungroomed hair-coat, piloerection, bradypnea, labored breathing pattern, dyspnea, breathing sounds, nostrils reddened, nasal discharge (serous), nostrils: red encrustations, nose/snout region: red encrustations, stridor, motility reduced, limp, high-legged gait, impaired reflexes, hypothermia, and decreased body weights. The duration of signs was governed by respiratory effects indicative of both lower and upper respiratory tract irritation and resolved towards the beginning of the second postexposure week. Necropsy findings were unremarkable. With regard to the respirability of the aerosol generated internationally recognized recommendations such as of SOT (1992) were fulfilled at large, i.e. the MMAD was 3.7-5.1 µm (GSD around 2.3).

On the basis of this study, the aerosolized test substance (solid aerosol) does not warrant classification as being harmful or toxic.

I. Material and methods

A. Materials:

1. Test Material:	BYI 08330
Description:	Technical grade, beige powder
Lot/Batch No.:	NLL 6425-9
Purity:	96.5 %
Compound Stability:	Stable at room temperature (~ 22 ºC)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1

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2. Vehicle and/or positive control:	Test substance was aerosolized as dust without the use of any additional vehicle
3. Test animals:	
Species:	Rat, males and females
Strain:	SPF bred Wistar [Hsd Cpb:WU (SPF)]
Age/weight at study initiation:	2 months / males: 183 g – 196 g, females: 158 g – 176 g
Source:	[redacted]
Acclimatisation:	Animals were acclimatized to the animal room conditions for at least 5 days before use
Housing:	During the acclimatization and study periods the animals were housed singly in conventional Makrolon® Type II cages (based on A. Spiegel and R. Gönnert, Zschr. Versuchstierkunde, 1, 38 (1961) and G. Meister, Zschr. Versuchstierkunde, 7, 144-153 (1965)).
Diet:	Ration consisted of a standard fixed-formula diet (NAFAG No. 9441 W10 pellets maintenance diet for rats and mice), *ad libitum*
Water:	Tap water, *ad libitum*
Environmental conditions:	**Temperature:** 22 ± 2 ºC **Humidity:** approximately 60 % **Lighting:** 12 h/12 h; artificial light from 6.00 a.m. to 6.00 p.m. Central European Time **Ventilation:** approximately 10 air changes per hour **Light intensity:** approximately 14 watt/m2 floor area

B. Study design and methods:

1. In life dates:

2. Animal assignment and treatment:

Five male and five female rats were nose-only exposed to mean solid aerosol concentrations of 1,100 and 4,183 mg/m^3 air. Body weights were measured before exposure, on days 3 and 7, and weekly thereafter. Individual weights were also recorded at death, if applicable. The period of observation was for 2 weeks. The appearance and behavior of each rat were examined carefully several times on the day of exposure and at least once daily thereafter. The rectal (colonal) temperatures were measured directly after cessation of exposure (approximately within labour after the end of exposure) using a Digimed digital thermometer with a rectal probe for rats. All rats were sacrificed at the end of the observation period using sodium pentobarbital (Narcoren®) (approximately 300 mg/kg body weight, intraperitoneal injection). All rats were given a gross-pathological examination. Consideration was given to performing a gross necropsy on animals as indicated by the nature of toxic effects, with particular reference to changes related to the respiratory tract. All gross pathological changes were recorded and evaluated.

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3. Generation of the test atmosphere / chamber description

Under dynamic conditions the test substance was fed into the intake of the cylindrical inhalation chamber. The test atmosphere was generated using a WRIGHT DUST FEEDER ( ). For dry powder dispersion, conditioned compressed air (28 liters/min) was used. The aluminium inhalation chamber has the following dimensions: inner diameter = 14 cm, outer diameter = 35 cm (two-chamber system), height = 25 cm (internal volume = about 3,8 l).

	Group 1	Group 2	Group 3
Target concentration (mg/m³)	control (air)	1,000	5,000
Actual concentration (mg/m³)	--	1,100.0	4,182.5
Temperature (mean, °C)	22.8	22.9	22.6
Relative humidity (mean, %)	<5.4	<7.1	<7.3
MMAD (µm)	--	3.68	5.13
GSD	--	2.29	2.37
Aerosol mass < 3 µm (%)	--	41.2	26.8
Mass recovered (mg/m³)	--	1,126.0	2,762.5

MMAD = Mass Median Aerodynamic Diameter,
-- = not applicable

II. Results and discussion:

Mortality:
No mortalities were observed.

Table 5.2.3-1 Doses, mortality / animals treated

Gravimetric concentration [mg/m³]	Toxicological results#			Duration of signs	Mortality [%]	Rectal temperature [°C]
Males						
0	0	0	0		0	37.4
1,100	0	4	5	0-5 d	0	35.1**
4,183	0	5	5	0-8 d	0	32.7**
LC_{50} (males): >4,183 mg/m³ air						
Females						
0	0	0	0		0	37.5
1,100	0	5	5	0-2 d	0	35.6**
4,183	0	5	5	0-8 d	0	33.5**
LC_{50} (females): >4,183 mg/m³ air						

1st figure = number of dead animals; 2nd figure = number of animals with signs 1h after cessation of exposure; 3rd figure = number of animals exposed; d = day; ** = p≤0.01

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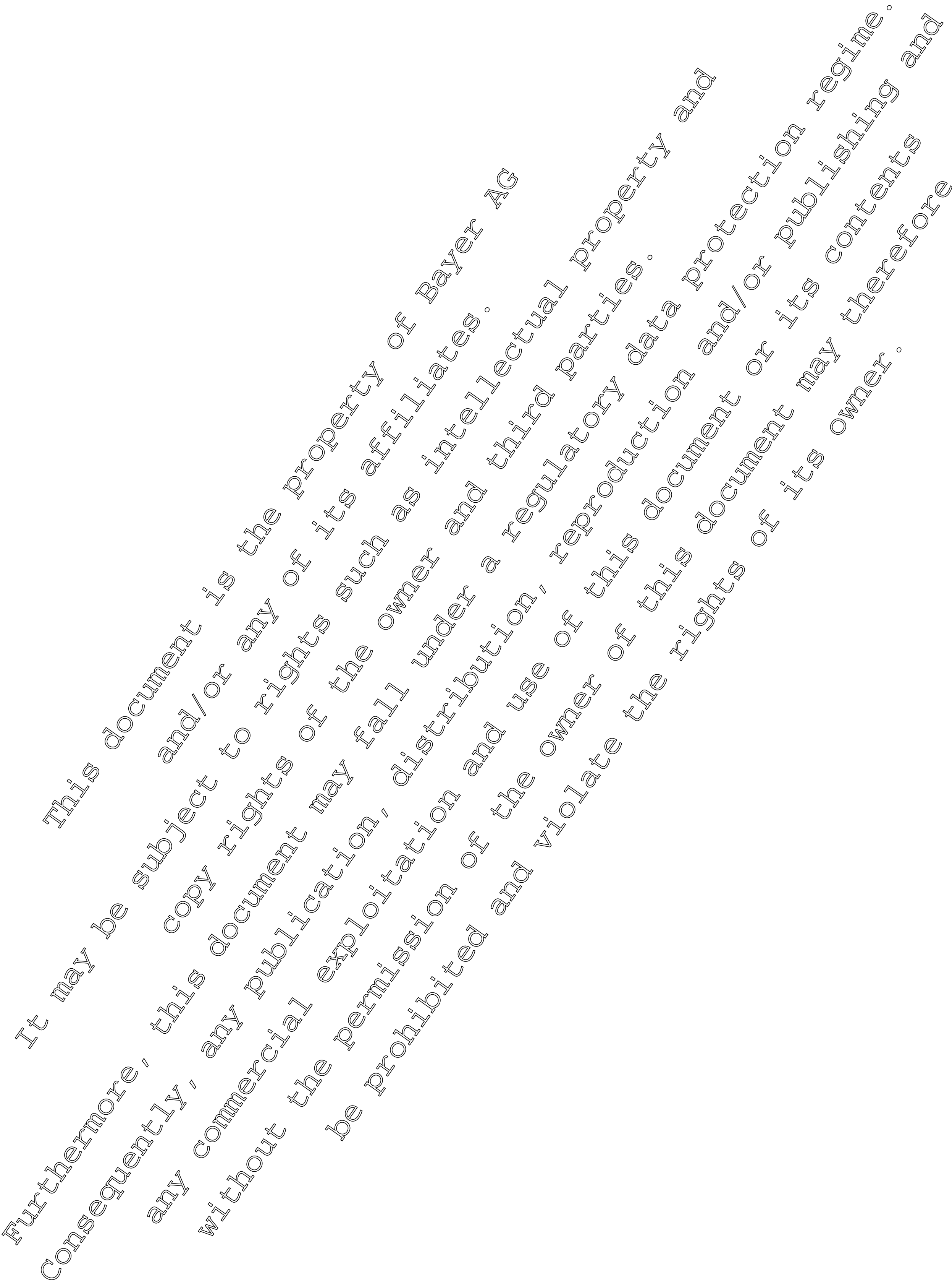
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Body weights:
Body weights were transiently decreased 3 days after exposure.
Clinical signs:
Clinical signs observed on days 0-8 were: ungroomed hair-coat, piloerection, bradypnea, labored breathing pattern, dyspnea, breathing sounds, nostrils reddened, nasal discharge (serous), nostrils: red encrustations, nose/snout region: red encrustations, stridor, motility reduced, limp, high-legged gait, impaired reflexes, hypothermia, and decreased body weights.
Necropsy:
No treatment-related findings.

III. Conclusion: BYI 08330 has a low acute inhalative toxicity in rats.
Classification/labelling according to Commission Directive 67/548/EEC: none
EPA Category IV

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IIA 5.2.4 Skin irritation

Report:	**KIIA 5.2.4/01, 2002**
Title:	Acute skin irritation test (patch test) of BYI 08330 in rabbits
Report No &	R 8147
Document No	M-062870-01-2
Guidelines:	OECD Guideline No. 404; EC Guideline B.4. Deviation(s): none
OECD/FIFRA GLPS	Yes. Deviation(s): none

Executive Summary

In a dermal irritation study, three male Himalayan rabbits, were exposed *via* the dermal route to 500 mg of BYI 08330 (purity 96.5%, batch No. NLL 6425-9) per animal. The test material was moistened with water, applied to the shorn dorsal skin (area: approx. 6 cm²) under semi-occlusive conditions for a duration of 4 hours. Animals were then observed for 3 days. None of the three animals exposed with BYI 08330 showed any test substance related lesions. In this study, BYI 08330 was not a dermal irritant. On the basis of this study, BYI 08330 does not warrant classification as being irritating to the skin.

I. Material and methods

A. Materials:

1. Test Material:	BYI 08330
Description:	Technical grade, beige powder
Lot/Batch No.:	NLL 6425-9
Purity:	96.5 %
Compound Stability:	Stable at room temperature (~22 °C)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1
2. Vehicle and/or positive control:	The test compound was mixed with 3 ml aqua ad iniectabilia
3. Test animals:	
Species:	Rabbit, males
Strain:	Himalayan
Age/weight at study initiation:	approx. 6.5 months / body weight at study start 2.2 kg
Source:	Germany
Acclimatisation:	at least 20 days

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Housing:	During the exposure period the animals were kept singly in special restrainers which allowed free movement of the head but prevented a complete body turn. Before and after the 4-hour exposure period the rabbits were kept separately in cages with dimensions of 425 mm x 600 mm x 380 mm.
Diet:	[redacted], D[redacted]
Water:	Tap water, *ad libitum*
Environmental conditions:	**Temperature:** 20°C ± 3°C **Humidity:** 55% ± 15% **Lighting:** darkend for periods of 12 hours **Light intensity:** 150 lux at approx. 1.5 m room height

B. Study design and methods:

1. In life dates: February 28, 2002 – March 3, 2002

2. Animal assignment and treatment:

Approximately 24 hours before the test, the fur was removed by shaving from the dorsal area of the trunk of the animals. Care was taken to avoid abrading the skin. Only animals with healthy intact skin were used. A dose of 500 mg was applied to the test site (area: approx. 6 cm²). Pulverised solids were moistened sufficiently with water to ensure good contact with the skin. The test substance was applied to the test site and then covered with a gauze patch. The patch was held in contact with the skin by means of a semi-occlusive dressing for the duration of the exposure period. The surrounding untreated skin served as a control. Exposure time was four hours. During the exposure the animals were kept in comfortable restrainers. The skin sites were evaluated immediately before the application of the test substance. After the 4-hour exposure period the patch was removed and the skin sites were evaluated. Scores were taken 60 minutes, 24, 48 and 72 hours after patch removal.

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II. Results and discussion:

Findings:
None of the three animals exposed with BYI 08330 showed any test substance related lesions. There were no systemic intolerance reactions.

Table 5.2.4-1 Summary of irritant effects (Score)

Animal no.	Skin irritation scores									
	before dosing		1h		24h		48h		72h	
	E	Oe	E	Oe	E	Oe	E	Oe	E	Oe
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0

0 = no pathological findings; E = erythema/eschar formation; h = hour; Oe = oedema formation

III. Conclusion: BYI 08330 has no irritant effect to the skin.
Classification/labelling according to Commission Directive 67/548/EEC: none
EPA Category IV

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IIA 5.2.5 Eye Irritation

Report:	**KIIA 5.2.4/01, [redacted] 2002**
Title:	Acute eye irritation study of BYI 08330 by instillation into the conjunctival sac of rabbits
Report No & Document No	R 8146 M-062864-01-3
Guidelines:	OECD Guideline No. 405; EC Guideline B.5. Deviation(s): none
OECD/FIFRA GLPS	Yes. Deviation(s): none

Executive Summary

In an eye irritation study, 100 mg of BYI 08330 (purity 96.5%, batch no. NLL 6425-9) was instilled into the conjunctival sac of the right eye of three young adult male Himalayan rabbits. Animals were then observed for the following 8 days. Irritation was scored using the Draize scheme for unwashed eyes.

Mild corneal opacity (grade 1) was observed in each of three rabbits at 24 hours resolving by day 7 or 8 postinstillation. Mild iritis (grade 1) was noted at 24 hours in two animals and at 72 hours postinstillation in the third animal, an effect that had resolved by day 6 or 7. Slight conjunctival redness was observed in each of three rabbits at 1 hour after dosing. The effect was resolved by day 4 or 5 postinstillation. Slight conjunctival chemosis was observed in one rabbit at 24 hours and which had resolved at 72 hours postinstillation.

In this study, BYI 08330 induced slight ocular irritation which was reversed during the study period. On the basis of this study, BYI 08330 warrant classification as being an eye irritant.

I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	Technical grade, beige powder
Lot/Batch No.:	NLL 6425-9
Purity:	96.5 %
Compound Stability:	Stable at room temperature (~ 22 ºC)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans:* 382608-10-8; *cis*: 203313-25-1

2. Vehicle and/or positive control: None

3. Test animals:

Species:	male rabbits
Strain:	Himalayan

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Age/weight at study initiation:	approx. 6.5 months / body weight at study start 2.3 kg – 2.5 kg
Source:	
Acclimatisation:	at least 20 days
Housing:	During the exposure period the animals were kept singly in special restrainers which allowed free movement of the head but prevented a complete body turn. Before and after the 4-hour exposure period the rabbits were kept separately in cages with dimensions of 425 mm x 600 mm x 380 mm.
Diet:	, D-
Water:	Tap water, *ad libitum*
Environmental conditions:	**Temperature:** 20°C ± 3°C **Humidity:** 55% ± 15% **Lighting:** darkend for periods of 12 hours **Light intensity:** 150 lux at approx. 1.5 m room height

B. Study design and methods:

1. In life dates: March 5, 2002 – March 14, 2002

2. Animal assignment and treatment:

A dose of 100 mg BYI 08330 was administered into the conjunctival sac of the right eye of rabbits after gently pulling the lower lid away from the eyeball. The lid was then gently held together for about one second in order to prevent loss of test material. The left eye, which remained untreated, served as a control. After the administration, the animals were kept separately in special restrainers which allowed free movement of the head but prevented a complete body turn, wiping of the eyes by the paws and excluded irritation of the eyes by excrements and urine. Examination of the eyes: the eyes were examined ophthalmoscopically with a slit lamp prior to the administration and also 1, 24, 48, 72 hours and 4 to 8 days after the administration. The eye reactions were observed and registered. Twenty-four (24) hours and 7 days after administration, the eyes were treated additionally with fluorescein and examined.

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II. Results and discussion

Findings:

Corneal opacity (grade 1) was observed in all animals 24 hours to 6 days after instillation, in animal nos. 1 and 2 until 7 days after instillation.

Irritation of the **iris** (grade 1) was observed in all animals:

- in animal no, 1: 24 hours to 6 days after instillation,
- in animal no. 2: 72 hours to 5 days after instillation and
- in animal no. 3: 24 hours to 5 days after instillation.

Conjunctival redness (grade 1) was observed in all animals 1 hour to 72 hours after instillation, in animal nos, 2 and 3 until 4 days after instillation. **Conjunctival chemosis** (grade 1) was noted in animal no. 3 from 24 to 48 hours after instillation. There were no systemic intolerance reactions,

Table 5.2.5-1 Summary of irritant effects (score)

Animal	Effects	1h	24 hrs	48 hrs	72 hrs	4 days	5 days	6 days	7 days	8 days
1	Corneal opacity	0	1	1	1	1	1	1	1	0
	Iris	0	1	1	1	1	1	1	0	0
	Redness conjunctivae	1	1	1	1	0	0	0	0	0
	Chemosis conjunctivae	0	0	0	0	0	0	0	0	0
2	Corneal opacity	0	1	1	1	1	1	1	1	0
	Iris	0	0	0	1	1	1	0	0	0
	Redness conjunctivae	1	1	1	1	1	0	0	0	0
	Chemosis conjunctivae	0	0	0	0	0	0	0	0	0
3	Corneal opacity	0	1	1	1	1	1	1	0	-
	Iris	0	1	1	1	1	1	0	0	-
	Redness conjunctivae	1	1	1	1	1	0	0	0	-
	Chemosis conjunctivae	0	1	1	0	0	0	0	0	-

- : not examined;

cornea:	0=no opacity;	1=scattered or diffuse areas of opacity, details of iris clearly visible
iritis:	0=normal;	1=markedly deepened rugae, swelling, moderate circumcorneal hyperaemia, or injection, iris still reacting to light
conjunct. redness:	0=blood vessels normal	1=some blood vessels injected
conj. chemosis	0= no swelling	1=any swelling above normal 2=obvious swelling with partial eversion of lids

III. Conclusion: BYI 08330 has an irritant effect to the eye.

Classification/labelling according to Commission Directive 67/548/EEC: yes (R 36)

EPA Category II

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IIA 5.2.6 Skin sensitization

Report:	**KIIA 5.2.6/01, ; 2002**
Title:	BYI 08330 – Study for the skin sensitization effect in guinea pigs (Guinea pig Maximization Test according to)
Report No & Document No	32273 M-076253-01-2
Guidelines:	OECD Guideline No. 406; EC Guideline B.6.; US EPA 712-C-98 197, OPPTS 870.2600. Deviation(s): none
OECD/FIFRA GLPS	Yes. Deviation(s): none

Executive Summary

In a dermal sensitization study, BYI 08330, purity: 96.5 %, batch No. NLL6425-9, was administered to 20 SPF-bred guinea pigs (strain Hsd Poc:DH). The test item was formulated in polyethylene glycol 400 to yield a suspension.

One animal died of unknown causes on day 15. The challenge with the 25% test item formulation led to skin effects (grade 1 - 3) in 18 of 19 animals (95%) in the test item group. No skin effects were noted in the control group animals. Appropriate historical control data using alpha hexyl cinnamic aldehyde demonstrated a positive response. On the basis of this study, BYI 08330 warrant classification as being a dermal sensitizer.

1. Test Material: BYI 08330; 3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro [4.5]dec-3-en-4-yl ethyl carbonate

Description: Technical grade, beige powder

Lot/Batch No.: NLL 6425-9

Purity: 96.5 %

Compound Stability: Stable at room temperature (~ 22 °C)

Chemical Name: 3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate

CAS No. of TGAI: *trans*: 382608-10-8; *cis*: 203313-25-1

2. Vehicle and/or positive control: Polyethylene glycol 400
Alpha hexyl cinnamic aldehyde

3. Test animals:

Species: Guinea pig, females

Strain: SPF-bred [Hsd Poc:DH]

Age/weight at study initiation: 5 – 6 weeks / body weight at study start 292 g – 366 g

Source: , Germany

Acclimatisation: at least five days before treatment

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Housing:	During the adaptation and study period the animals were conventionally kept in type IV Makrolon® cages, in groups of five during the adaptation period and in groups of two or three per cage throughout the study period. The cages were exchanged for ones with clean bedding at least two times per week. Low-dust wood shavings supplied by [redacted] [redacted], were used as bedding.	
Diet:	[redacted] Switzerland	
Water:	tap water	
Environmental conditions:	**Temperature:**	22 +/- 3°C (possibly drifting higher at outdoor temperatures above 24°C)
	Humidity:	40-60 %
	Lighting:	Twelve hours; artificial lighting from 6 AM to 6 PM
	Air changes:	>= 10 times per hour

B. Study design and methods:

1. In life dates: April 23, 2002 – May 17, 2002

2. Animal assignment and treatment:

20 animals were used for the test item group and 10 control animals. The test item was formulated in polyethylene glycol 400 to yield a suspension. The volume applied per injection site was 0.1 ml (intradermal induction) and 0.5 ml for each topical application (topical induction and topical challenge).
The test item concentrations were as follows:
- intradermal induction: 5 %,
- topical induction: 50 %,
- topical challenge: 25 %.

The skin reactions were assessed 48 and 72 hours after the start of the application to induce the challenge and for the range-finding studies to establish concentrations for the topical induction and challenge in accordance with the following pattern: 0 = No reaction; 1 = Slight localized redness; 2 = Moderate confluent redness; 3 = Severe redness and swelling. The animals were observed for clinical signs at least once daily throughout the entire study period. The body weights of the animals were recorded before initiating the study and at the end of the study.

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II. Results and discussion:

Findings:

One animal of the test item group (animal no. 27) died on day 15 of the study. At the end of the study, the mean body weight of the treatment group animals was in the same range than that of the control group animals.

The challenge with the 25% test item formulation led to skin effects (grade 1 - 3) in 18 of 19 animals (95%) in the test item group and no skin effects were seen in the control group animals.

Table 5.2.6-1 Number of animals exhibiting skin effects

	Test item group (19 animals)					Control group (10 animals)				
	test item patch			control patch		test item patch			control patch	
Hrs	***48***	***72***	***total***	***48***	***72***	***48***	***72***	***total***	***48***	***72***
Challenge 25 %	**18**	**18**	**18**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

III. Conclusion: BYI 08330 has a skin-sensitization potential under the conditions of the Maximization test.

Classification/labelling according to Commission Directive 67/548/EEC: Xi, R 43 (may cause sensitization by skin contact).

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Report:	**KIIA 5.2.6/02, 2004**
Title:	BYI 08330 – Study for the skin sensitization effect in guinea pigs (Buehler Patch Test)
Report No &	AT01317
Document No	M-078494-01-2
Guidelines:	OECD Guideline No. 406; EC Guideline B.6.; US EPA 712-C-98 197, OPPTS 870.2600; Deviation(s): none
OECD/FIFRA	Yes.
GLPS	Deviation(s): Analytical determinations of the stability of the paste (71%) in polyethylene glycol 400 for administration were not performed. This deviation did not limit the assessment of the results.

Executive Summary

In a dermal sensitization study, BYI 08330, purity: 97.2 %, mixed batch no. 08045/0014, was administered to 20 SPF-bred guinea pigs (strain Crl:HA). The test item was formulated in polyethylene glycol 400 to yield a suspension or a paste.
There was no dermal response to either induction (71% BYI 08330) or challenge (71% BYI 08330) applications. Appropriate historical control data using alpha hexyl cinnamic aldehyde demonstrated a positive response. On the basis of this study, BYI 08330 does not warrant classification as being a dermal sensitizer.

1. Test Material:	BYI 08330; 3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro [4.5]dec-3-en-4-yl ethyl carbonate
Description:	Technical grade, beige powder
Lot/Batch No.:	mixed batch no. 08045/0014
Purity:	97.2 %
Compound Stability:	Stable at room temperature (~ 22°C)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1
2. Vehicle and/or	Polyethylene glycol 400
positive control:	Alpha hexyl cinnamic aldehyde
3. Test animals:	
Species:	Guinea pig, females
Strain:	SPF-bred [Crl:HA]
Age/weight at study initiation:	4-5 weeks / body weight at study start 309 g – 375 g
Source:	Germany
Acclimatisation:	at least five days before treatment

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Housing:	During the adaptation and study period the animals were conventionally kept in type IV Makrolon® cages, in groups of five during the adaptation period and in groups of two or three per cage throughout the study period. The cages were exchanged for ones with clean bedding at least two times per week. Low-dust wood shavings supplied by [redacted] [redacted], were used as bedding.	
Diet:	[redacted], Switzerland	
Water:	tap water	
Environmental conditions:	**Temperature:**	22 +/- 3°C (possibly drifting higher at outdoor temperatures above 24°C)
	Humidity:	40-70 %
	Lighting:	Twelve hours; artificial lighting
	Air changes:	>= 10 times per hour

B. Study design and methods:

1. In life dates: May 18, 2004 – June 17, 2004

2. Animal assignment and treatment:

20 animals were used for the test item group and 10 as control animals. The test item was formulated in polyethylene glycol 400 to yield a suspension. The volume applied per animal was 0.5 ml vehicle in the control group and 500 mg test item mixed with 0.2 ml vehicle in the test item group.
The test item concentrations were as follows:
- 1st to 3rd induction: 71 %,
- challenge: 71 %.

The skin reactions were assessed 30 hours after initiation of the induction exposures, and 30 and 54 hours after the beginning of the challenge with the following scoring system: 0 = No reaction; 1 = Slight localized redness; 2 = Moderate confluent redness; 3 = Severe redness and swelling. The animals were observed for clinical signs at least once daily throughout the entire study period. The body weights of the animals were recorded by using a Mettler Toledo scale with printer LC-P45 on day 1 before the first induction, and after the last evaluation on day 31 in the control group.

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II. Results and discussion:

Findings:

Appearance and behaviour of the test item group were not different from the control group. In the control and test item group there were no skin effects during the induction treatments. By the end of the study the mean body weight of the treatment group animals was in the same range than that of the control group.

Table 5.2.6-2 Number of animals exhibiting skin effects

	Test item group (20 animals)					Control group (10 animals)				
	test item patch			control patch		test item patch			control patch	
Hrs	***30***	***54***	***total***	***30***	***54***	***30***	***54***	***total***	***30***	***54***
Challenge 71 %	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

III. Conclusion: BYI 08330 has no skin-sensitization potential under the conditions of the Buehler test.

Classification/labelling according to Commission Directive 67/548/EEC: None

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Report:	**KIIA 5.2.6/03, 2004**
Title:	BYI 08330 – Evaluation of potential dermal sensitization in the Local Lymph Node Assay
Report No &	SA04120
Document No	M-090707-01-2
Guidelines:	OECD Guideline 429 (2002). Deviation(s): none
OECD/FIFRA GLPS	Yes. Deviation(s): none

Executive Summary

In a Local Lymph Node Assay, BYI 08330, purity: 97.2 %, mixed batch no. 08045/0014, was administered to female mice (strain CBA/J) at a concentration of 10, 5, 2.5 or 1% (vehicle: Dimethylformamide) on external surfaces of each ear (i.e. 25 µl/ear) for three consecutive days (days 0, 1 and 2).
Induction of a 3.4-fold increase in lymph node cell proliferation compared with concurrent vehicle controls was noted at a treatment concentration of 1% BYI 08330. The positive control group using Isoeugenol demonstrated a proliferation index value of 3.4 at a treatment concentrations of 5%.
BYI 08330 has a skin-sensitization potential under the conditions of the Local Lymph Node Assay.

1. Test Material:	BYI 08330
Description:	Technical grade, beige powder
Lot/Batch No.:	08045/0014
Purity:	97.2 %
Compound Stability:	Stable at room temperature (~ 22 °C)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1
2. Vehicle and/or positive control:	Dimethylformamide Isoeugenol
3. Test animals:	
Species:	Mouse, females
Strain:	CBA/J
Age/weight at study initiation:	8 weeks / body weight at study start approximately 22 g – 23 g
Source:	France
Acclimatisation:	at least five days before study start
Housing:	The animals were housed in a room within a barrier maintained unit with restricted entry. Mice were housed individually in suspended, stainless steel, wire-mesh cages.
Diet:	Certified rodent pellet diet: AO4C-10, , France)
Water:	tap water

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Environmental conditions:	**Temperature:**	20°C - 24°C
	Humidity:	40 - 70%
	Lighting:	12-hour light, 12-hour dark cycles (7 am - 7 pm)
	Air changes:	The Animal Unit ventilation system is constantly monitored; regular checks are made in the individual animal room for the target specification of 10 to 15 air changes per hour.

B. Study design and methods:

1. In life dates: June 2, 2004 – June 7, 2004

2. Animal assignment and treatment:

Forty five female CBA/J mice were allocated to 9 groups of five animals each:

- four groups receiving the test substance at a concentration of 10, 5, 2.5 or 1%,
- four positive control groups receiving the reference substance (Isoeugenol) at a concentration of 5, 2.5, 1 or 0.5%,
- one control group receiving the vehicle, Dimethylformamide (DMF).

The test substance, positive control and the vehicle were applied on external surfaces of each ear (i.e. 25 µl/ear) for three consecutive days (days 0, 1 and 2) at the appropriate concentration.
On day 5, the cell proliferation in the local lymph nodes was measured by incorporation of tritiated methyl thymidine and the obtained values were used to calculate proliferation indices

II. Results and discussion:

Findings:
No mortality and no clinical signs were observed during the study. No cutaneous reactions were observed in the vehicle, reference control or treated groups. The proliferation index values of the test substance were 5.9, 5.4, 4.3 and 3.4 at treatment concentrations of 10, 5, 2.5 and 1% respectively. The proliferation index values of the positive control were 3.4, 1.8, 1.3 and 0.8 at treatment concentrations of 5, 2.5, 1 and 0.5% respectively.

III. Conclusion: BYI 08330 has a skin-sensitization potential under the conditions of the Local Lymph Node Assay.
Classification/labelling according to Commission Directive 67/548/EEC: Xi, R 43 (may cause sensitization by skin contact).

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IIA 5.2.7 Potentiation/interactions of multiple active substances or products

Not required.

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IIA 5.3 Short-term toxicity

Summary of short-term toxicity

Type of study	Animal species	Doses tested	NOAEL	LOAEL
Oral, feeding, 28 days	rat	0 - 500 - 5,000 ppm	5,000 ppm (f) (=502 mg/kg bw/day)	None
Oral, gavage, 41 days,(parent) (mechanistic)	rat	0 - 1,000 mg/kg	None	1,000 mg/kg
Oral, gavage, 21 days, (enol) (mechanistic)	rat	0 - 800 mg/kg	None	800 mg/kg
Oral, feeding, 28 days	mouse	0 -500 - 5,000 ppm	5,000 ppm (m) (=1415 mg/kg bw/day)	None
Oral, feeding, 28 days	dog	0 - 100 - 400 - 1,600 - 6,400 ppm	1,600 ppm (m/f) (=42 / 70 mg/kg bw/day)	6,400 ppm (m/f) (=104/127 mg/kg bw/day)
Oral, feeding, 90 days	rat	0 - 150 - 600 - 2,500 - 10,000 ppm	2,500 ppm (m/f) (=148 / 188 mg/kg bw/day)	10,000 ppm (m/f) (=616/752 mg/kg bw/day)
Oral, feeding, 90 days	mouse	0 - 70 - 350 - 1,700 - 7,000 ppm	7,000 ppm (m/f) (=1305/1515 mg/kg bw/day)	None
Oral, feeding, 90 days	dog	0 - 150 - 300 - 1,200 - 4,000*/2,500 ppm	2,500 ppm (m / f) (=81 / 72 mg/kg bw/day)	4,000 ppm (a.i. intake not calculated)
Oral, feeding, 1 year	dog	0-200-600-1,800 ppm	600 / 1,800 ppm (m / f) (=20 / 48 mg/kg bw/day)	1,800 / None (m/f) (=55 mg/kg bw/day)
Dermal, 28 days	rat	0 - 100 - 300 - 1,000 mg/kg bw	Systemic: 1,000 mg/kg bw Local 1,000 mg/kg bw	None

* dose reduced to 2,500 ppm after 2 weeks of treatment

BYI 08330 belongs to the chemical class of cyclic ketoenoles and acts as an ACCase (Acetyl CoA Carboxylase) inhibitor. In eukaryotes and prokaryotes, ACCase is a key enzyme in fatty acid biosynthesis. The biological activity of cyclic ketoenoles correlates with inhibition of lipogenesis in treated insects, resulting in decreased lipid contents (notably triglycerides and free fatty acids), inhibition of the ability of younger insects to develop through the various growth stages, and ultimately c[illegible]inating in a diminished capacity of the insect to reproduce as adults.

The insecticidal mode of action is not reflected by the results of the short-term toxicological studies in rodents and dogs. Rats, mice and dogs did not exhibit changes in plasma lipid parameters such as plasma triglycerides and plasma cholesterol. In male rats, BYI 08330 induced germ cell toxicity at very high dose levels, accompanied by reduced body weight development. The testicular effects proved to be reversible after cessation of treatment. Mice and dogs showed no testicular effects.

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The primary target in rats is the germinal epithelium of the testes: Tubular degeneration and vacuolization of epithelial cell layers of seminiferous tubules was noted at the end of the 90-day (subchronic) feeding study at the highest dose level of 10,000 ppm (equal to 616 mg/kg bw/day). As a consequence, abnormal spermatozoa and hypospermia was detected also in the epididymis at 10,000 ppm. Reversibility of these effects was assessed at the highest dose tested in the subchronic rat study (10,000 ppm). No testicular and epididymal effects have been noted histopathologically after 90 days of treatment at 2,500 ppm (equal to 148 mg/kg bw/day), indicating a low potency of BYI 08330 to trigger this effect in rats.
Other effects in subchronically treated rats were limited to declines in terminal body weight (8.4%) and body weight gain (17.6%) in 10,000 ppm male rats and an increased incidence of accumulation of alveolar macrophages in both sexes at 10,000 ppm. A NOAEL of 2,500 ppm (equal to 148 / 188 mg/kg bw/day in males / females) was established in the 90-day feeding study in rats. Reproductive toxicity studies (see IIA 5.6) confirmed sperm cell toxicity after repeated high doses with an abrupt transition from no-adverse-effect-levels to adverse-effect-levels indicating a steep dose-response for BYI 08330-induced testicular toxicity.

Unlike the rat no effects of any kind emerged in the mouse, which was tested up to the limit dose of 7,000 ppm (equal to 1,305 / 1,515 mg/kg bw/day in males / females) in a 90-day feeding study. Although male mice were dosed in excess of the limit dose, no testicular lesions were detectable after 90 days of treatment. *In vitro* results from a Liverbead™ study using hepatocytes from male rats, mice, and humans (Report No. SA05319) revealed species differences in the metabolization of BYI 08330. Specifically, mouse and human hepatocytes, unlike the rat, were able to conjugate the enol via UDP-GT, thus reducing the systemic burden of the enol which has been shown to be the toxophore responsible for the degenerative testicular effects in the rat (for further details see IIA 5.6).

In the dog, treatment-related testicular toxicity was not observed in any study up to the highest dose tested (i.e. 28-day feeding study: 6,400 ppm equal to 104 mg/kg/bw/day; 90-day feeding study: 2,500 ppm equal to 81 mg/kg/bw/day; chronic feeding study: 1,800 ppm equal to 55 mg/kg/bw/day). Body weight declines and reduced food consumption were noted at 6,400 ppm in the 28-day feeding study and body weight reductions at 4,000 ppm in the 90-day feeding study, requiring a reduction of the high dose to 2,500 ppm after two weeks of treatment in the 90-day feeding study.

Treatment-related non-adverse declines in circulating thyroid hormones were identified exclusively in the dog. In the subacute dog study, Thyroxine (T4) was decreased in 1,600- and 6,400-ppm males and in 6,400-ppm females. Triiodothyronine (T3) was also decreased at 6,400 ppm in both genders. In the subchronic dog study, T4 was decreased in both sexes in 1,200- and 2,500-ppm animals and T3 in 2,500-ppm animals. In the chronic dog study, T4 was decreased in both genders in 600- and 1,800-ppm animals and T3 in 1,800-ppm males.

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However, no changes in thyroid weight, thyroid pathology, no compensating increases in thyrotropin (TSH), and no clinical observations (e.g. neurological signs) suggestive of thyroid compromise and/or hypothyroidism were detected in either sex up to the highest dose tested. In the chronic dog study, a slight reduction in the size of peripheral thyroid follicles was noted in two high dose male dogs at 1,800 ppm (equal to 55 mg/kg bw/day). The normal canine thyroid consists of follicles of varying size. In general, smaller follicles with cuboidal follicular epithelium are located in the central regions of the gland extending to larger follicles (three to four times larger) often with flattened squamous follicular epithelium at the periphery. These morphological changes could indicate a reduction in the amount of colloid present within the gland, however, all treated animals had decreases in T4. Therefore, it is questionable, if this change in these two males may correlate with the clinical chemistry decreases of mean values for T4 which occurred in treated males and females.

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IIA 5.3.1 Oral 28-day toxicity

Rat

Report:	**KIIA 5.3.1/01, & ; 1998**
Title:	Cyclic ketoenols BSN 3457, BSN 2342, FHN 7504, FHN 8330 - Subacute exploratory toxicity studies in rats (application by feed over 4 weeks)
Report No &	T0061869
Document No	M-040236-01-2
Guidelines:	not according to a definite guideline; intended as dose range-finding study only
OECD/FIFRA	No
GLPS	

Executive Summary

In an exploratory feeding study, several structurally-related test compounds had been administered separately to different animals. In the present report for all test compounds, only the results for BYI 08330 (named as FHN 8330) are reported. Groups of 5 female Wistar rats of the strain Hsd/Win:WU were dosed with BYI 08330, purity 98.2%, batch no. FHN 8330-3, at dietary concentrations of 0, 500 or 5,000 ppm over a period of 4 weeks. Dietary concentrations were equal to 0, 47.3 or 501.8 mg/kg bw/day. The control group consisted of 10 female animals. The following parameters were determined: clinical signs, food consumption, body weights, clinical chemistry (aspartate aminotransferase, alkaline phosphatase, γ-glutamyltransferase, plasma total cholesterol and triglycerides), gross necropsy, liver weight and liver histopathology. Furthermore, tissue samples of liver were investigated for a potential proliferative effect of BYI 08330.
Dietary exposure to BYI 08330 resulted in no treatment-related effects in female rats up to a dietary concentration of 5,000 ppm.

II. Results and Discussion:

General observations: No treatment-related clinical signs or mortalities were observed. Body weight development and feed intake was not affected.

Clinical chemistry: Not affected.

Liver cell proliferation: No relevant increase in liver cell proliferation and no effect on nuclear area values.

Gross necropsy, liver weights: No treatment-related macroscopic changes were seen. Liver weight was not affected by treatment.

Histopathology: No treatment-related histopathological findings were observed in the liver.

III. Conclusions: The subacute toxicity study in female rats established a NOAEL of 5,000 ppm (equal to 502 mg/kg bw/day), based on the lack of treatment-related findings at 5,000 ppm.

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Mouse

Report:	**KIIA 5.3.1/02; [redacted] 2001**
Title:	BYI 08330: Subacute study with mice
Report No &	T2070951
Document No	M-035927-01-2
Guidelines:	not according to a definite guideline; intended as dose range-finding study only
OECD/FIFRA GLPS	No, intended as dose range-finding study only

Executive Summary

In an exploratory feeding study, BYI 08330, batch no. NLL 6425-7, purity: 97.0 %, was administered to groups of 5 male Crl:CD-1(ICR)BR mice at dietary concentrations of 0, 500, and 5,000 ppm over a period of up to 28 days. Dietary concentrations were equal to 0, 136.5 and 1,415 mg/kg bw/day. Animals were regularly inspected and weighed, food intake was determined. Activity of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase was measured in peripheral blood, as well as cholesterol and triglyceride concentrations. Organs (adrenals, liver, testes and epididymides) were weighed and subjected to gross pathological and histopathological examinations. Dietary exposure to BYI 08330 resulted in no treatment-related effects in male mice up to a dietary concentration of 5,000 ppm.

Results and Discussion:

General observations: No treatment-related clinical signs or mortalities were observed. Body weight development was not affected. A slightly lower feed intake in both treatment groups was not considered compound-related, because the changes were not correlated with the dose.

Clinical chemistry: Determination of activity of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and cholesterol and triglyceride concentrations in peripheral blood did not reveal treatment-related effects.

Gross necropsy, liver weights: Organ weights of adrenals, liver, testes and epididymides did not differ significantly from the control weights. Necropsy revealed no treatment-related findings.

Histopathology: Histopathological investigations of liver/gall bladder, adrenal glands, testes and epididymides revealed no evidence of any dose-related histopathological changes in animals up to and including 5,000 ppm.

III. Conclusions: The subacute toxicity study in male mice established a NOAEL of 5,000 ppm (equal to 1,415 mg/kg bw/day), based on the lack of treatment-related findings at 5,000 ppm.

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Dog

Report:	**KIIA 5.3.1/03 [redacted]; 2004**
Title:	Technical Grade BYI 08330: A Subacute Toxicity Feeding Study in the Beagle Dog
Report No &	201012
Document No	M-182239-01-1
Guidelines:	Dose-range finder with reference to OECD 409 (MAY 1981), US-EPA-OPPTS 870.3150 (1998)
OECD/FIFRA GLPS	Yes (certified laboratory). Deviations: none

Executive Summary

In a dose range finding study, BYI 08330, batch no.: NLL 6425, purity: 93.5 - 96.5 %, was administered for 28 – 29 days to two male and two female Beagle dogs per group at dietary concentrations of 0, 100, 400, 1,600, and 6,400 ppm (equal to 0, 3, 13, 42 or 104 mg/kg bw/day for males and 0, 3, 12, 70 or 127 mg/kg bw/day for females, respectively).
Treatment-related findings were restricted to body weight reduction and food consumption declines in 6,400-ppm animals and thyroid hormone declines in the 1,600 and 6,400 ppm dose group males and the 6,400 ppm dose group females.
The NOAEL of 1,600 ppm (equal to 42 or 70 mg/kg bw/day in males or females, respectively) in the subacute toxicity feeding study in dogs is based on a body weight decrease at 6,400 ppm.

Results and Discussions:

General observations: There was a marked decrease in body weight and food consumption in the 6,400 ppm dose group of either sex. Clinical observations were limited to a thin body state beginning at day 15 in males and females in the 6,400 ppm group. Neurological examinations revealed a mild hind-limb wheelbarrowing deficit at 6,400 ppm in one female and muscular atrophy associated with reduced postural reactions in the other female and in one male of this high-dose group. These alterations were not considered to be specific target-organ effects of the compound, but were thought to be secondary to the emaciation and thinness observed in these animals. This was confirmed microscopically by the lack of any degenerative nervous system changes in these animals.

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Table 5.3.1-1: Mean Body Weights (g)

Study Day	Dose Group (ppm)				
	0	100	400	1,600	6,400
Males					
0	**9952.5**	**1,0681.5**	**1,0777.5**	**9,125.0**	**9,355.0**
7	**1,0040.0**	**1,0534.5**	**1,0614.0**	**8,934.0**	**8,424.5**
14	**1,0268.0**	**1,1319.5**	**1,1317.5**	**9,265.5**	**8,189.0**
21	**1,0964.0**	**1,1701.0**	**1,1134.5**	**9,694.5**	**7,843.0**
28 #	**1,1230.0**	**1,1421.0**	**1,2398.0**	**9,830.5**	**7,542.0**
Gain	**1,277.5**	**739.5**	**1,620.5**	**705.5**	**-1,813.0**
Females					
0	**7,662.0**	**8,136.0**	**7,962.0**	**7,287.5**	**7,664.5**
7	**7,352.0**	**7,863.5**	**7,802.0**	**7,010.0**	**6,766.5**
14	**7,863.5**	**8,696.5**	**8,717.5**	**7,799.0**	**6,788.0**
21	**8,320.0**	**8,600.0**	**8,496.0**	**7,900.0**	**6,603.5**
28 #	**8,596.0**	**8,991.5**	**8,928.0**	**7,871.5**	**6,143.5** *
Gain	**934.0**	**855.5**	**966.0**	**584.0**	**-1,521.0**

* = $p < 0.05$; # terminal body weight

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Table 5.3.1-2: Food consumption (g/animal/day)

Study Day	Dose Group (ppm)				
	0	100	400	1,600	6,400
			Males		
1	**464.5**	**458.0**	**374.0**	**214.0**	**260.5**
2	**441.0**	**368.0**	**474.5**	**209.5**	**147.0**
3	**440.0**	**306.0**	**377.0**	**218.5**	**50.0**
4	**376.5**	**393.0**	**359.5**	**261.0**	**63.5**
5	**463.0**	**410.5**	**482.5**	**298.0**	**105.0**
6	**460.5**	**420.0**	**313.5**	**360.0**	**116.5**
7	**209.0**	**150.5**	**167.5**	**111.0**	**119.5**
14	**472.5**	**447.5**	**463.5**	**298.5**	**192.0**
21	**487.0**	**484.5**	**392.5**	**345.0**	**189.0**
28	**471.0**	**435.0**	**536.5**	**293.5**	**169.0**
29	**386.0**	**200.0**	**379.0**	**336.0**	**176.0**
			Females		
1	**324.0**	**287.0**	**326.0**	**199.5**	**67.0**
2	**217.5**	**309.0**	**197.0**	**335.5**	**117.0**
3	**347.0**	**313.0**	**383.5**	**379.5**	**82.5**
4	**307.0**	**246.0**	**288.5**	**391.0**	**108.5**
5	**202.5**	**303.5**	**227.5**	**429.0**	**299.5**
6	**334.5**	**367.5**	**318.5**	**447.0**	**309.0**
7	**116.0**	**151.5**	**125.5**	**232.0**	**176.0**
14	**243.5**	**304.5**	**308.0**	**427.5***	**125.5***
21	**376.0**	**355.5**	**298.5**	**504.5**	**198.5**
28	**361.0**	**397.5**	**217.0**	**368.0**	**153.0**
29	**337.0**	**245.0**	**429.0**	**371.5**	**108.0**

* = $p < 0.05$

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Hematology, urinalysis: No treatment-related findings.

Clinical chemistry: There were statistical and nonstatistical downward trends in thyroid hormones (T_4) in the 1,600 and 6,400 ppm dose group males and the 6,400 ppm dose group females which were considered compound related. Triiodothyronine (T_3) was also decreased at 6,400 ppm in both genders. However, these alterations were not considered to be biologically significant due to the lack of accompanying abnormalities in the thyroid weights or histopathological appearance of the thyroid gland in dogs demonstrating hormonal alterations.
Statistically significant declines in T_4 at 400 ppm and in T_3 in males at 400 and 1,600 ppm were small and are most probably attributed to the inherent biological variation. The low TSH levels especially in 6,400 ppm females are most likely due to chance variation, since the baseline level was low in this group, and when considering the high variability for this endpoint (see also 90-day feeding study). There was no effect on UDP-glucuronyltransferase activity. Other compound-related changes consisted of decreases in the calcium and albumin levels of the 6,400 ppm dogs, and were considered secondary to emaciation. Calcium and albumin levels are known to be interrelated, for calcium tends to decrease in hypoalbuminemic states (Duncan, J. R. "Endocrine System" In: *Veterinary Laboratory Medicine*, Clinical Pathology, 2nd edition, Iowa State University Press, Ames, Iowa, 1986, pp 181-200)

Table 5.3.1-3: Mean thyroxine (T 4) levels

Parameter	Dose Group (ppm)									
	0		100		400		1,600		6,400	
Males										
T 4 day -11	2.6		2.3		2.1		2.4		2.6	
T 4 day 7	1.8		1.7		1.2		0.8		0.3	
T 4 day 23	1.5		1.5		0.9	*	0.6	*	0.3	*
Females										
T 4 day -11	3.5		2.9		2.3		3.5		2.2	
T 4 day 7	1.9		1.6		0.9		1.2		0.4	
T 4 day 23	1.9		1.7		1.0		1.4		0.3	*

* = $p < 0.05$

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Table 5.3.1-4: Mean thyroxine (T 3) levels

Parameter		Dose Group (ppm)									
		0		100		400		1,600		6,400	
Males											
T 3	day -11	1.0		0.9		0.9		1.1		0.9	
T 3	day 7	1.0		1.0		0.8	*	0.7	*	0.4	*
T 3	day 23	0.9		0.9		0.7		0.6		0.4	
Females											
T 3	day -11	0.8		0.7		0.8		1.1	*	0.8	
T 3	day 7	1.1		0.7		0.9		1.0		0.5	
T 3	day 23	1.0		0.8		0.9		1.0		0.4	*

* = $p < 0.05$

Table 5.3.1-5: Mean TSH levels

Parameter		Dose Group (ppm)									
		0		100		400		1,600		6,400	
Males											
TSH	day -11	0.32		0.21		0.31		0.21		0.16	
TSH	day 7	0.24		0.16		0.22		0.09		0.07	
TSH	day 23	0.24		0.10		0.13		0.12		0.06	
Females											
TSH	day -11	0.34		0.09		0.25		0.24		0.12	
TSH	day 7	0.34		0.17		0.20		0.19		0.03	
TSH	day 23	0.24		0.13		0.22		0.29		0.02	

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Ophthalmology: No treatment-related effects.

Gross necropsy, organ weights, histopathology: Emaciation and thinness were noted in one male and in both of the female dogs of the 6,400 ppm group, which were considered secondary to compound administration. Reduced thymus and decreases in thymic weight were observed, which were considered to reflect a non-specific stress response stemming from the dogs´general, overall state of emaciation / thinness at the high dose level. Terminal body weight was decreased in male and female dogs at 6,400 ppm. The lower terminal body weight mean in 1,600 ppm males was probably related to the lower baseline mean.
Histopathology displayed exacerbated sexual immaturity in one high-dose group male, which was considered secondary to the animals overall state of emaciation, rather than a specific target-organ effect. This animal weighed approximately 4 kg less than the control males and had also a mild diffuse atrophy of the parotid salivary glands, which may have reflected the animal´s decreased food intake.

Conclusion: The NOAEL of 1,600 ppm (equal to 42 or 70 mg/kg bw/day in males or females, respectively) in the subacute toxicity feeding study in dogs is based on decreased body weights, food consumption declines, and an overall state of emaciation at 6,400 ppm (equal to 104 or 127 mg/kg bw/day in males or females). No marked toxicity was determined in the present study.
The NOEL of 400 ppm (equivalent to 13 mg/kg bw/day in males) is based on nonadverse declines in circulating thyroxine (T4) levels at 1,600 ppm, equivalent to 42 mg/kg bw/day.

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IIA 5.3.2 Oral 90-day toxicity (rodents)

Rat

Report:	**KIIA 5.3.2/01, [redacted]; 2005**
Title:	Technical Grade BYI 08330, A Subchronic Toxicity Testing Study in the Rat
Report No & Document No	201136 M-252787-01-1
Guidelines:	OECD 408 (1995), US-EPA-OPPTS 870.3100 (1998), JMAFF 12-Nou-san-No. 8147, 2-1-9 (2000). Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a 90-d feeding study, BYI 08330, batch no.: NLL 6425-9; purity 96.5% - 98.1 %, was administered for approximately 14 weeks to 10 male and 10 female Wistar Hanover rats (strain: Crl:WI [Glx/BRL/Han] IGS BR) with their diet at concentrations of 0, 150, 600, 2,500 or 10,000 ppm. The dietary concentrations were equal to doses of 0, 9, 36, 148, and 616 mg/kg bw/day in males and to 0, 11, 46, 188, and 752 mg/kg bw/day in females, respectively. In order to determine the reversibility of possible effects, additional recovery groups (0 and 10,000 ppm) with an equal number of rats were observed for additional 4 weeks without treatment. In addition to standard guideline parameters, the following hepatic enzyme investigations were performed in all animals per group at the end of the treatment: N-Demethylase, O-Demethylase, UDP-Glucuronosyltransferase, and hepatic Cytochrome P-450 content.

Dietary exposure to BYI 08330 resulted in a 17.6% decline in body weight gain and a 8.4% decline in terminal body weight in males at 10,000 ppm. Organ weight changes were limited to a slight decrease in absolute testicular weight at 10,000 ppm. Histopathology revealed an increased incidence of minimal to severe abnormal spermatozoa and hypospermia in the epididymis, and minimal to moderate tubular degeneration in the testis at 10,000 ppm. An increased incidence of minimal to slight accumulation of alveolar macrophages was noted in both sexes at 10,000 ppm. At the end of the recovery period (approximately 4 weeks), in every affected parameter reversibility was observed with many parameters fully recovering to control levels.

The NOAEL for this study is 2,500 ppm, equivalent (m/f) to 148 / 188 mg/kg bw/day, respectively, and is based on the following findings at 10,000 ppm (equivalent (m/f) to doses of 616 / 752 mg/kg bw/day): reduced body weight, decreased absolute testicular weight, testicular tubular degeneration, abnormal epididymal spermatozoa and hypospermia, and increased accumulation of alveolar macrophages in both sexes.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	Technical grade, beige powder
Lot/Batch No.:	NLL 6425-9
Purity:	96.5% (12/2001); 93.5% (6/2002); 93.1% (1/2003)
Compound Stability:	Stable at room temperature (~ 22 ºC)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1

2. Vehicle and/or positive control: None

3. Test animals:

Species:	Rat, males and females (nulliparous and non-pregnant)
Strain:	Wistar Hanover rats (Crl:WI[Glx/BRL/Han]IGS BR)
Age/weight at study initiation:	Approximately 9 weeks old / 263.4 - 269.6 g mean group weight males; 171.9 - 177.2 g mean group weight females
Source:	
Housing:	Individually housed in suspended stainless steel wire-mesh cages, each containing a feeder, a source of water (pressure-activated water nipples), and deotized (sanitized) cage board in the bedding tray
Diet:	Diet 5002 in "meal" form; *ad libitum*
Water:	Municipal water supply of Kansas City, MO; *ad libitum*. Exception: Control animals were without pressurized water for approximately 1.5 days (between nominal Days 91 and 93). Only the water remaining in the lines would have been available during this time.

Environmental conditions:	**Temperature:**	18 to 26 ºC
	Humidity:	30 to 70% (relative)
	Air changes:	12 air changes per day (daily average)
	Photoperiod:	12 hr of light [7:00 a.m. to 7:00 p.m.] alternating with 12 hr of darkness; lights toggled off during ophthalmic examinations
Acclimatisation:	03/25/02 (receipt) - 04/01/02 (release for study)	

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B. Study design:

1. In life dates: Start: 04/01/02 (released for study); 04/09/02 (initiation of exposure)
End: 07/18/02 (terminal sacrifice complete; subchronic animals)
08/13/02 (terminal sacrifice complete; recovery animals)

2. Animal assignment:
04/02/02; assigned to a control or one of four chemically-treated groups using a weight stratification-based computer program obtained from INSTEM Computer Systems (Stone, Staffordshire, UK). Each animal on study was identified with a microchip (, DE) subcutaneously implanted on their dorsal surface in the region between the scapulae; the chip was encoded with a unique number, specifying the animal's sex, dose group, cage number, and study affiliation.

Test Group	Conc. in Diet (ppm)	Dose to Animal (mg/kg; x±Std)	Male	Female
Control (A)	0	0	20	20
Low (B)	150	males 8.9 ± 1.4 females 11.4 ± 1.2	10	10
Mid 1 (C)	600	males 35.9 + 5.0 females 46.1 ± 4.5	10	10
Mid 2 (D)	2,500	males 147.9 + 23.2 females 188.3 ± 24.2	10	10
High (E)	10,000	males 615.9 ± 91.6 females 752.0 ± 84.6	20	20

3. Dose selection rationale:
Doses were selected based principally upon the toxicological profile which emerged in the female rat over the course of a subacute (4-week) toxicity testing study conducted with the test article at doses of 0, 500, and 5,000 ppm BYI 08330 (Report No. T0061869). In that study, findings directly attributable to exposure to the test substance were not observed. Therefore, doses of 0, 150, 600, 2,500, and 10,000 ppm were chosen at which to conduct the present study. It was anticipated that the low and high doses chosen of 150 and 10,000 ppm would constitute NOAEL and maximum tolerated doses, respectively, with the intermediate doses of 600 and 2,500 ppm providing confirmation of any dose-response relationships that may have emerged.

4. Diet preparation and analysis:
The test compound was mixed directly with the feed; control diet consisted of untreated feed. Samples of each batch of feed mixed were 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 and stored under freezer conditions until presented to the animals the following week.

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BYI 08330 was administered at a constant concentration in the feed for the duration of the study. The concentration of the AI of the test substance in the various test diets was analytically verified from samples of each dietary level taken during weeks 1, 2, 3, 7, and 11. In addition, one recovery sample was analyzed with each group of samples during the test concentration analyses. The homogeneity and stability of BYI 08330 when mixed in the dietary carrier was characterized concurrently with the in-life phase of the study.
To evaluate the uniformity of distribution/homogeneity of the test substance in the diet, an analysis of feed containing nominal concentrations of 70 and 10,000 ppm BYI 08330 was conducted. Three (3) samples were taken for analysis from each of 3 distinct layers (top, middle, bottom; 9 samples total) in a Hobart mixing bowl. A homogeneous distribution of the test substance in the feed was defined in terms of a coefficient of variation (CV), derived from the 9 samples taken, which was ≤ 10%.
To evaluate the stability of the test substance in the diet, a time and temperature analysis of feed containing nominal concentrations of 70 and 10,000 ppm BYI 08330 was conducted. Stability in the feed was assessed following 1, 3, and 7 days of room temperature storage (~22 ºC) and 7, 14, and 28 days of freezer storage (~ -23 ºC). The stability analysis was carried out as follows: An initial sample was taken immediately after the test substance was mixed with the ration. Each batch of ration, mixed at the 2 concentrations, was then divided into 2 portions. One portion was placed in a room temperature environment, the other in the freezer. Feed samples which remained at room temperature were sampled for analysis on Days 0, 1, and 7; feed samples which remained in the freezer were sampled for analysis on Days 7, 14, and 28. Chemical stability in the ration was defined in terms of that period of time that 80% of the test substance, relative to the initial concentration, was recoverable. The liquid chromatographic methodology used to perform the dietary analyses (i.e., homogeneity, stability, and concentration verifications) required for this study has been described previously.

Results:

Homogeneity Analysis: The mean concentrations of BYI 08330 in the feed, sampled from 3 distinct layers in the mixing bowl and containing a nominal concentration of either 70 or 10,000 ppm, were determined to be 68.7 ppm (range 65.0-73.1 ppm; CV = 3.2%) and 9,445 ppm (range 8,817-9,943 ppm; CV = 3.2%), respectively. Based on a CV ≤ to 10%, BYI 08330 was judged to be homogeneously distributed in the feed over a concentration range of 70-10,000 ppm.

Stability Analysis: Following 7 days of room temperature storage, the mean analytically-determined concentration of the AI of the test substance in the 70- or 10,000-ppm admixture was determined to be 68.5 ppm (63.3 ppm on Day 0) and 9,564 ppm (9,405 ppm on Day 0), respectively. Following 28 days of freezer storage, the mean analytically determined concentration of the AI of the test substance in the 70- and 10,000-ppm admixtures was determined to be 71.4 ppm and 9,544 ppm, respectively (63.3 and 9,405 ppm, respectively on Day 0). BYI 08330 mixed in rodent ration was judged to be stable at room temperature for at least 7 days and following freezer storage for a minimum 28 days, over a concentration range of 70-10,000 ppm.

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Concentration Analysis: Mean analytical concentrations for each dose group were 143, 596, 2,431, and 9,904 ppm, ranging from 95-99% of the corresponding nominal concentrations of 150, 600, 2,500, and 10,000 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 99% and ranged from 95-102% for rodent ration spiked with 150, 600, 2,500 or 10,000 ppm BYI 08330.

5. Statistics:

Continuous data that were examined statistically were evaluated initially for equality or homogeneity of variance using Bartlett's test. Group means were further analyzed by a one-way analysis of variance (ANOVA) followed by Dunnett's test. In the event of unequal variances, and at the discretion of the Study Director, data were subject to non-parametric procedures consisting of a Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test for between-group comparisons. Frequency data were initially examined for trends; data suggestive of a potential effect were then statistically evaluated using the Chi-Square, Fisher Exact, or Chi-Square and Fisher Exact tests. On a case-by-case basis, and at the discretion of the study director, data were subject to additional statistical procedures other than those mentioned above. For the Bartlett test, a probability (p) value < 0.001 was considered significant; for all other statistical tests, differences with p values ≤ 0.05 were considered statistically significant. All statistical evaluations were performed using software obtained from either INSTEM Computer Systems or SAS Institute Inc..

C. Methods:

1. Observations:

1a. Cageside Observations:

For approximately 18 weeks of the study (14 weeks for subchronic group animals; 18 weeks for recovery animals), a general assessment of all remaining animals was carried out at least daily (generally twice daily during the normal work week and once daily on weekends and holidays). These assessments were generally with respect to moribundity and mortality, but also included a more general clinical observation for readily apparent clinical signs (in addition to moribundity and mortality).

1b. Clinical Examinations:

Once every week for approximately 18 weeks of the study, detailed physical examinations for clinical signs of toxicity were performed on all surviving animals, which included evaluation of external surface areas, orifices, posture, general behavior, respiration, and excretory products.

1c. Neurological Evaluations:

Parameters specifically designed to evaluate possible neurological effects were not included in this study (i.e., other than those effects that would be identified during cage-side or detailed observations). These types of investigations were conducted as a part of other toxicology studies, including an acute neurotoxicity screening study (Report No. 201283) and a 1-year chronic rat study (Report No. 201486).

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2. Body weight:
Individual body weight determinations were performed weekly throughout the in-life phase of the study on all surviving animals.

3. Food consumption and compound intake:
Individual food consumption determinations were performed weekly throughout the in-life phase of the study on all surviving animals (g consumed/animal/day and g consumed/kg body wt/day). In addition, using specifically defined criteria, food consumption data were corrected, as conditions dictated, to account for misleading indications of food intake (i.e., excessive spillage, clogged feeders, etc.). Test substance intake (mg/kg body wt/day) was calculated using weekly mean 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

4. Ophthalmologic examination:
Ophthalmologic exams were conducted on all acclimatized animals prior to exposure, and then again on all surviving animals just prior to their respective termination dates (i.e., recovery animals were not evaluated at the end of the exposure phase, only at the end of the recovery phase). For listing of the defining conditions of the 5-level severity grading system in use at this facility for assigning severity grades to both ophthalmologic and nonneoplastic histopathologic lesions, see the "Histology" section below.

5. Hematology & Clinical Chemistry:
Blood was collected from all surviving animals just prior to their respective termination dates (i.e., non-recovery animals following 13 ± 1 weeks of study; recovery animals following 17 ± 1 weeks on study). Blood for standard serum chemistry and hematological determinations was drawn via the orbital sinus following an overnight fast under light anesthesia (IsoFlo®; Isoflurane). An additional aliquot of blood (non-fasted) for prothrombin time determinations was taken immediately preceding necropsy via cardiac puncture of animals under CO_2 anesthesia (diaphragm was cut following blood collection).

5a. Hematology:

X	Hematocrit (Hct)*	X	Leukocyte differential count*
X	Hemoglobin (Hgb)*	X	Mean corpuscular Hgb (MCH)*
X	Leukocyte count (WBC)*	X	Mean corpuscular Hgb concentration (MCHC)
X	Erythrocyte count (RBC)*	X	Mean corpuscular volume (MCV)*
X	Platelet count (PLTS)*	X	Reticulocyte count (Retic)
X	Blood clotting measurements*	X	Erythrocyte morphology
	(Thromboplastin time)	X	Heinz bodies (HZ)
	(Clotting time)		
X	Prothrombin time (PT)		

* Recommended for 90-day oral rodent studies based on Guideline 870.3100
The CHECKED (X) parameters were examined.

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5b. Clinical Chemistry:

	ELECTROLYTES		OTHER
X	Calcium (Calc)	X	Albumin (Alb)*
X	Chloride (Cl)	X	Creatinine (Creat)*
	Magnesium	X	Blood urea nitrogen (Urea-N)*
X	Phosphate (Phos)	X	Total Cholesterol (Chol)*
X	Potassium (K)*	X	Globulin (Glob)
X	Sodium (Na)*	X	Glucose, fasting (Gluc)*
	ENZYMES	X	Total bilirubin (T-Bili)
X	Alkaline phosphatase (ALP)*	X	Total protein (T-Prot)*
	Cholinesterase (ChE)	X	Triglycerides (Trig)
X	Creatine phosphokinase (CK)		Serum protein electrophoresis
X	Lactic acid dehydrogenase (LDH)	X	Uric acid (Uric-A)
X	Alanine aminotransferase (ALT)*	X	Thyroxine (T4)
X	Aspartate aminotransferase (AST)*	X	Triiodothyronine (T3)
	Sorbitol dehydrogenase*	X	Thyroid Stimulating Hormone (TSH)
X	Gamma-glutamyltransferase (GGT)*		
	Glutamate dehydrogenase		
X	Cytochrome P-450, hepatic (Cyto P-450)		
X	N-Demethylase, hepatic (N-Demeth)		
X	O-Demethylase, hepatic (O-Demeth)		
X	UDP-Glucuronosyltransferase, hepatic (UDP-GT)		

* Recommended for 90-day oral rodent studies based on Guideline 870.3100
The CHECKED (X) parameters were examined.

6. Urinalysis:

Urine was collected from all surviving animals just prior to their respective termination dates (i.e., non-recovery animals following 13 ± 1 weeks on study; recovery animals following 17 ± 1 weeks of study). The urine was collected on an overnight basis (timed) from non-fasted animals.

X	Appearance*	X	Glucose (Glu)
X	Volume (UVol)*	X	Ketones (Ket)
X	Specific gravity (Sp.Gr.)*	X	Bilirubin (Bil)
X	pH (pH)*	X	Leukocytes (U-Leu)
X	Sediment (microscopic)	X	Blood (Bld)*
X	Protein (Pro)*	X	Nitrite (Nit)
		X	Urobilinogen (UROB)

1 Optional for 90-day oral rodent studies
* Recommended for 90-day oral rodent studies
The CHECKED (X) parameters were examined.

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7. Sacrifice and Pathology:

7a. Gross pathology:

All animals were sacrificed by CO_2 asphyxiation (or CO_2 anesthesia followed by clipping of the diaphragm) and subject to a postmortem examination, which included (1) documenting and saving all gross lesions, (2) weighing of the designated organs, and (3) collecting representative tissue specimens for histopathologic evaluation. Tissues were preserved (nominally in 10% buffered formalin) and selected organs were weighed and organ/body weight ratios calculated. Following collection of a liver specimen for histopathological analysis, the remaining liver tissue from at least 8 surviving animals (at approximately 14 and 18 weeks) was snap frozen in liquid nitrogen, pending further biochemical analyses.

	DIGESTIVE SYSTEM		**CARDIOVASC./HEMAT.**		**NEUROLOGIC**
X	Bile duct (rat)	X	Aorta*	XX	Brain*+
X	Cecum*	X	Bone marrow*	X	Cerebellum
X	Colon*	XX	Heart*+	X	Cerebrum-Midbrain
X	Duodenum*	X	Lymph node, cervical*	X	Eyes*
X	Esophagus*	X	Lymph node, mesenteric*	X	Medulla/Pons
	Gall bladder (not rat)*	XX	Spleen*+	X	Nerve, optic*
X	Ileum*	XX	Thymus*+	X	Nerve, sciatic*
X	Jejunum*			X	Pituitary*
XX	Liver*+		**UROGENITAL**	X	Spinal cord, cervical*
X	Pancreas*	X	Cervix	X	Spinal cord, lumbar*
X	Rectum*	X	Clitoral gland^	X	Spinal cord, thoracic*
X	Salivary glands*	XX	Epididymides*+		
X	Stomach, glandular*	XX	Kidneys*+		**OTHER**
X	Stomach, non-glandular*	X	Mammary gland*	X	Bone, femur
X	Tongue	XX	Ovary*+	X	Bone marrow
X	Tooth	X	Preputial gland^	X	Bone, rib/cc jct
		X	Prostate*	X	Bone, sternum
	RESPIRATORY	X	Seminal vesicle*	X	Gross lesions and masses*
X	Larynx*	XX	Testicle*+	X	Harderian gland
XX	Lung*	X	Urinary bladder*	X	Joint, fem/tib
X	Nasal structure	XX	Uterus*+	X	Muscle, protocol
X	Nasopharynx*	X	Vagina*	X	Physical Identifier (ID chip)
X	Oral structure			X	Skin, protocol*
X	Trachea*		**GLANDULAR**	X	Zymbal's gland^
		XX	Adrenal gland*+		
		X	Exorbital/lacrimal gland^		
		X	Parathyroid*		
		XX	Thyroid*		

* Recommended for 90-day oral rodent studies based on Guideline 870.3100
+ Organ weights required for rodent studies.
The CHECKED (X) tissues were collected for histological examination. The organs CHECKED (XX) were also weighed. The organs CHECKED (^) were preserved for possible micropathologic evaluation only.

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7b. Micropathology:
With the exception of the physical identifier (microchip), the vagina, and the exorbital/lacrimal, clitoral, preputial, and Zymbal glands (of which the latter 5 were taken and preserved for possible micropathologic evaluation), representative sections of the tissues shown in Table 5 (previous page) were processed for all control (0 ppm) and high-dose (10,000 ppm) animals, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under the light microscope. Tissues in which compound-related effects were detected were examined at lower doses as necessary to establish no-observed-effect levels. Histopathological and ophthalmological lesions were assigned a semi-quantitative ranking of the severity grade/stage from "Normal (within normal limits)" to the "most severe case (Grade 5).

II. Results and Discussion:

Findings:
General observations: No treatment-related clinical signs or mortalities were observed. Body weight gain was reduced at 10,000 ppm in male rats. Difference to controls was 17.6% at the end of the study; a corresponding decline of 8.4% was noted in absolute body weight at termination of treatment. No relevant effect on feed intake was determined.

Table 5.3.2-1: Affected body weight and weight gain

Parameter	Dose Group (ppm)				
	0	150	600	2,500	10,000
Males					
Body weight week -1	**236.0**	**236.5**	**238.0**	**235.7**	**235.5**
Body weight week 1	**269.6**	**263.6**	**267.6**	**263.7**	**263.4**
Body weight week 7	**387.2**	**382.9**	**375.8**	**379.2**	**364.7**
Body weight week 14	**446.7**	**432.9**	**423.3**	**439.2**	**409.3**
Total weight gain (g)	**177.1**	**169.3**	**156.0**	**175.5**	**145.9** *
Weight gain (%) of controls		**96**	**88**	**99**	**82**

* = $p < 0.05$

Hematology: Not affected.

Clinical chemistry: Not affected.

Urinalysis: Not affected.

Hepatic enzyme profile: Not affected.

Ophthalmology: No treatment-related effects.

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Gross necropsy, organ weights: No treatment-related macroscopic changes were seen. With the exception of a slight decrease in absolute testis weight at 10,000 ppm, absolute and relative organ weights showed no treatment-related changes.

Histopathology:

Epididymis: At the end of the treatment, an increased incidence of minimal to severe abnormal spermatozoa and hypospermia were noted in the epididymides of 10,000-ppm males. Multi-nucleated giant cells, spherocytes (immature sloughed germ cells), and tissue debris (recorded as abnormal spermatozoa) were present within the duct of the epididymis well above that found in control males. In addition, the quantity of sperm within the ducts was reduced (hypospermia). Only one recovery male each showed abnormal spermatozoa or hypospermia at the end of the 4-week recovery period suggesting reversibility of lesions.

Testes: A minimal to moderate tubular degeneration was noted in the testes of 10,000-ppm males. Tubular degeneration and vacuolization in the 10,000 ppm dose group was generally multifocal in distribution, and occurred in the germinal epithelial layer of seminiferous tubules with degeneration and loss of epithelial cells. This finding correlated with the slight decrease in absolute testicular weight. Only one recovery male showed tubular degeneration in the testes at the end of the 4-week recovery period suggesting reversibility of lesions.

Table 5.3.2-2: Average incidence and severity of treatment-related microscopic findings

	Main Dose Group (ppm)										Recovery Group (ppm)			
	0		150		600		2,500		10,000		0		10,000	
Males														
Epididymis, abnormal spermatozoa	**0**		**-**		**0**		**0**		**9 (1.8)**	*	**0**		**1 (1.0)**	
Epididymis, hypospermia	**0**	*	**-**		**0**		**0**		**5 (2.6)**	*	**0**		**1 (5.0)**	
Testes, tubular degeneration	**0**		**-**		**0**		**0**		**5 (2.0)**	*	**0**		**1 (2.0)**	
Testes, vacuolization	**0**		**-**		**0**		**0**		**5 (1.6)**	*	**0**		**0**	
Lungs, alveol. macrophages	**2 (1.0)**		**4 (1.3)**		**-**		**5 (1.4)**		**9 (1.3)**	*	**3 (1.7)**		**7 (1.3)**	
Females														
Lungs, alveol. macrophages	**1 (1.0)**		**-**		**-**		**1 (1.0)**		**7 (1.9)**		**4 (1.3)**		**5 (1.4)**	

* = $p < 0.05$

Lungs: An increased incidence of minimal to slight accumulation of alveolar macrophages was noted in both sexes at 10,000 ppm. Alveolar macrophage accumulation is a common background lesion in the rat. The increases in the 10,000 ppm male and female dose levels, however, were considered to be

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affected by compound administration. At the end of the recovery period, reversibility was suggested for the affected parameter.

III. Conclusions: The subchronic toxicity study in rats established a NOAEL of 2,500 ppm (equal to 148 or 188 mg/kg bw/day) in males or females, respectively, based on the following findings at 10,000 ppm (equal to 616 mg/kg bw/day in males and 752 mg/kg bw/day in females, respectively): reduced body weight, decreased absolute testicular weight, testicular tubular degeneration, abnormal epididymal spermatozoa and hypospermia, and increased accumulation of alveolar macrophages in both sexes.

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Mouse

Report:	**KIIA 5.3.2/02, [redacted]; 2005**
Title:	Technical Grade BYI 08330, A Subchronic Toxicity Testing Study in the Mouse
Report No & Document No	201284 M-255359-01-1
Guidelines:	None. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a 90-day feeding study, BYI 08330, batch no.: batch no.: NLL 6425-9, purity 96.5% - 93.1%, was administered for approximately 14 weeks to 15 male and 15 female CD-1 mice with their diet at concentrations of 0, 70, 350, 1,700 or 7,000 ppm. The dietary concentrations were equal to doses of 0, 12.8, 59.6, 300, and 1,305 mg/kg bw/day in males and to 0, 16.0, 72.4, 389, and 1,515 mg/kg bw/day in females, respectively.
No effects attributable to exposure to BYI 08330 were observed at any dose level tested. The male and female LOAEL for this study was not established (>7,000 ppm). The NOAEL is 7,000 ppm, equivalent (m/f) to 1,305 / 1,515 mg/kg bw/day, respectively.

I. Material and methods:

A. Materials:

1. Test Material:	BYI 08330
Description:	Technical grade, beige powder
Lot/Batch No.:	NLL 6425-9
Purity:	96.5% (12/2001); 93.5% (6/2002); 93.1% (1/2003)
Compound Stability:	Stable at room temperature (~ 22 ºC)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1

2. Vehicle and/or positive control: None

3. Test animals:	
Species:	Mouse; males and females (nulliparous and non-pregnant)
Strain:	CD-1 [ICR]/BR
Age/weight at study initiation (Day 0):	Approximately 9 weeks old / 30.1 - 30.9 g mean group weight males; 25.3 - 25.9g mean group weight females
Source:	[redacted]

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Housing: Individually housed in suspended stainless steel wire-mesh cages, each containing a feeder, a source of water (pressure-activated water nipples), and deotized (sanitized) cage board in the bedding tray (animals passing initial shipment exam were co-housed during a portion of the Acclimatisation)

Diet: Diet 5002 in "meal" form; *ad libitum*

Water: Municipal water supply of Kansas City, MO; *ad libitum*. Exception: Some animals were without pressurized water for approximately 1.5 days (between nominal Days 40 and 42). Only the water remaining in the lines would have been available during this time.

Environmental conditions:
Temperature: 19 to 25 °C
Humidity: 30 to 70% (relative)
Air changes: 15 air changes per day (daily average)
Photoperiod: 12 hr of light [7:00 a.m. to 7:00 p.m.] alternating with 12 hr of darkness

Acclimatisation: 05/13/02 (receipt) - 05/20/02 (release for study)

B. Study design:

1. In life dates: Start: 05/20/02 (released for study); 05/30/02 (initiation of exposure)
End: 09/06/02 (terminal sacrifice complete)

2. Animal assignment: 05/22/02; assigned to a control or one of four chemically-treated groups using a weight stratification-based computer program obtained from INSTEM Computer Systems (Stone, Staffordshire, UK). Each animal on study identified with a microchip (, DE) subcutaneously implanted on their dorsal surface in the region between the scapulae; the chip was encoded with a unique number, specifying the animal's sex, dose group, cage number, and study affiliation.

Test Group	Conc. in Diet (ppm)	Dose to Animal (mg/kg; x±Std)	Male	Female
Control (A)	0	0	15	15
Low (B)	70	males 12.8 ± 0.9 females 16.0 ± 1.3	15	15
Mid 1 (C)	350	males 59.6 + 4.3 females 72.4 ± 5.4	15	15
Mid 2 (D)	1,700	males 300 + 28 females 389 ± 31	15	15
High (E)	7,000	males 1,305 + 86 females 1,515 ± 102	15	15

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3. Dose selection rationale:

Doses were selected based principally upon the toxicological profile which was observed in the mouse over the course of a subacute (4-week) toxicity testing study conducted with the test article at doses of 0, 500, and 5,000 ppm BYI 08330 (Report No. T2070951). In that study, findings directly attributable to the exposure to the test substance were not observed. Therefore, doses of 0, 70, 350, 1,700, and 7,000 ppm were chosen at which to conduct the present study. It was anticipated that the low and high doses chosen of 70 and 7,000 ppm would constitute NOAEL and maximum tolerated doses, respectively, with the intermediate doses of 350 and 1,700 ppm providing confirmation of any dose-response relationships that may have emerged.

4. Diet preparation and analysis:

The test compound was mixed directly with the feed; control diet consisted of untreated feed. Samples of each batch of feed mixed were 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 every other week and stored under freezer conditions until presented to the animals.

BYI 08330 was administered at a constant concentration in the feed for the duration of the study.

The concentration of the AI of the test substance in the various test diets was analytically verified from samples of each dietary level for all batches prepared. In addition, one recovery sample was analyzed with each group of samples during the test concentration analyses. The homogeneity and stability of BYI 08330 when mixed in the dietary carrier was characterized prior to the experimental start of the study (10,000 ppm; 70 ppm homogeneity). The stability analysis of the 70-ppm ration was determined just following the experimental start of the study.

To evaluate the uniformity of distribution/homogeneity of the test substance in the diet, an analysis of feed containing nominal concentrations of 70 and 10,000 ppm BYI 08330 was conducted. Three (3) samples were taken for analysis from each of 3 distinct layers (top, middle, bottom; 9 samples total) in a Hobart mixing bowl. A homogeneous distribution of the test substance in the feed was defined in terms of a coefficient of variation (CV), derived from the 9 samples taken, which was ≤ 10%.

To evaluate the stability of the test substance in the diet, a time and temperature analysis of feed containing nominal concentrations of 70 and 10,000 ppm BYI 08330 was conducted. Stability in the feed was assessed for up to 7 days of room temperature storage (~22 ºC) and up to 28 days of freezer storage (~-23 ºC). The stability analysis was carried out as follows: An initial sample was taken immediately after the test substance was mixed with the ration. Each batch of ration, mixed at the 2 concentrations, was then divided into 2 portions. One portion was placed in a room temperature environment, the other in the freezer. Feed samples which remained at room temperature were sampled for analysis on Days 0, 1, 3 or 4, and 7; feed samples which remained in the freezer were sampled for analysis on Days 7, 14 or 15, and 28. Chemical stability in the ration was defined in terms of that period of time that 80% of the test substance, relative to the initial concentration, was recoverable.

The liquid chromatographic methodology used to perform the dietary analyses (i.e., homogeneity, stability, and concentration verifications) required for this study has been described previously.

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Results:

Homogeneity Analysis: The mean concentrations of BYI 08330 in the feed, sampled from 3 distinct layers in the mixing bowl and containing a nominal concentration of either 70 or 10,000 ppm, were determined to be 68.7 ppm (range 65.0-73.1 ppm; CV = 3.2%) and 9,445 ppm (range 8,817-9,943 ppm; CV = 3.2%), respectively. Based on a CV ≤ to 10%, BYI 08330 was judged to be homogeneously distributed in the feed over a concentration range of 70-10,000 ppm.

Stability Analysis: Following 7 days of room temperature storage, the mean analytically-determined concentration of the AI of the test substance in the 70- or 10,000-ppm admixture was determined to be 68.5 ppm (63.3 ppm on Day 0) and 9,564 ppm (9,405 ppm on Day 0), respectively. Following 28 days of freezer storage, the mean analytically determined concentration of the AI of the test substance in the 70- and 10,000-ppm admixtures was determined to be 71.4 ppm and 9,544 ppm, respectively (63.3 and 9,405 ppm, respectively on Day 0). BYI 08330 mixed in rodent ration was judged to be stable at room temperature for at least 7 days and following freezer storage for a minimum 28 days, at a concentration range of 70-10,000 ppm.

Concentration Analysis: Mean analytical concentrations for each dose group were 70.9, 328, 1,665, and 6,753 ppm, ranging from 94-101% of the corresponding nominal concentrations of 70, 350, 1,700, and 7,000 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 100% and ranged from 98-102% for rodent ration spiked with 70, 350, 1,700, and 7,000 ppm BYI 08330.

5. Statistics:
Continuous data that were examined statistically were evaluated initially for equality or homogeneity of variance using Bartlett's test ([redacted], 1967). Group means were further analyzed by a one-way analysis of variance (ANOVA) followed by Dunnett's test. In the event of unequal variances, and at the discretion of the Study Director, data were subject to non-parametric procedures consisting of a Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test for between-group comparisons. Frequency data were initially examined for trends; data suggestive of a potential effect were then statistically evaluated using the Chi-Square, Fisher Exact, or Chi-Square and Fisher Exact tests. On a case-by-case basis, and at the discretion of the study director, data were subject to additional statistical procedures other than those mentioned above. For the Bartlett test, a probability (p) value < 0.001 was considered significant; for all other statistical tests, differences with p values ≤ 0.05 were considered statistically significant. All statistical evaluations were performed using software obtained from either INSTEM Computer Systems or SAS Institute Inc..

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C. Methods:

1. Observations:

1a. Cageside Observations:

For approximately 14 weeks of the study, a general assessment of all remaining animals was carried out at least daily (generally twice daily during the normal work week and once daily on weekends and holidays). These assessments were generally with respect to moribundity and mortality, but also included a more general clinical observation for readily apparent clinical signs (in addition to moribundity and mortality).

1b. Clinical Examinations:

Once each week for approximately 14 weeks of the study, detailed physical examinations for clinical signs of toxicity were performed on all surviving animals, which included evaluation of external surface areas, orifices, posture, general behavior, respiration, and excretory products.

1c. Neurological Evaluations:

Parameters specifically designed to evaluate possible neurological effects were not included in this study (i.e., other than those effects that would be identified during cage-side or detailed observations). These types of investigations were conducted as part of other toxicology studies, including an acute neurotoxicity screening study and a 1-year chronic rat study.

2. Body weight:

Individual body weight determinations were performed weekly throughout the in-life phase of the study on all surviving animals (with the exception of 1 animal on Day 49).

3. Food consumption and compound intake:

Individual food consumption determinations (g consumed/animal/day and g consumed/kg body wt/day) were performed weekly throughout the in-life phase of the study on all surviving animals (with the exception of 1 animal on Day 14). In addition, using specifically defined criteria, food consumption data were corrected, as conditions dictated, to account for misleading indications of food intake (i.e., excessive spillage, clogged feeders, etc.). Test substance intake (mg/kg body wt/day) was calculated using weekly mean 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

4. Ophthalmological examination:

Ophthalmological examinations were not included in this study. Ophthalmical examinations were included as part of a subchronic investigation with the test substance in rats.

5. Hematology & Clinical Chemistry:

Following approximately 13 weeks on study, blood was collected from all surviving animals just prior to termination. Blood for standard serum chemistry and hematological determinations was drawn via the orbital sinus; non-fasted. Due to blood volume considerations, only selected serum chemistry

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evaluations were included. A full serum chemistry profile was included as a part of a subchronic investigation with the test substance in rats (Report No. 201136).

5a. Hematology:

X	Hematocrit (Hct)*	X	Leukocyte differential count*
X	Hemoglobin (Hgb)*	X	Mean corpuscular Hgb (MCH)*
X	Leukocyte count (WBC)*	X	Mean corpuscular Hgb concentration (MCHC)
X	Erythrocyte count (RBC)*	X	Mean corpuscular volume (MCV)*
X	Platelet count (PLTS)*	X	Reticulocyte count (Retic)
	Blood clotting measurements*	X	Erythrocyte morphology
	(Thromboplastin time)	X	Heinz bodies (HZ)
	(Clotting time)	X	Red cell distribution width (RDW)
	Prothrombin time (PT)	X	Hemoglobin distribution width (HDW)

* Recommended for 90-day oral rodent studies based on Guideline 870.3100
The CHECKED (X) parameters were examined.

5b. Clinical Chemistry:

	ELECTROLYTES		**OTHER**
	Calcium (Calc)		Albumin (Alb)*
	Chloride (Cl)	X	Creatinine (Creat)*
	Magnesium	X	Blood urea nitrogen (Urea-N)*
	Phosphate (Phos)	X	Total Cholesterol (Chol)*
	Potassium (K)*		Globulin (Glob)
	Sodium (Na)*		Glucose, fasting (Gluc)*
	ENZYMES		Total bilirubin (T-Bili)
X	Alkaline phosphatase (ALP)*		Total protein (T-Prot)*
	Cholinesterase (ChE)	X	Triglycerides (Trig)
	Creatine phosphokinase (CK)		Serum protein electrophoresis
	Lactic acid dehydrogenase (LDH)		Uric acid (Uric-A)
X	Alanine aminotransferase (ALT)*		Thyroxine (T4)
X	Aspartate aminotransferase (AST)*		Triiodothyronine (T3)
	Sorbitol dehydrogenase*		Thyroid Stimulating Hormone (TSH)
	Gamma-glutamyltransferase (GGT)*		
	Glutamate dehydrogenase		
	Cytochrome P-450, hepatic (Cyto P-450)		
	N-Demethylase, hepatic (N-Demeth)		
	O-Demethylase, hepatic (O-Demeth)		
	UDP-Glucuronosyltransferase, hepatic (UDP-GT)		

* Recommended for 90-day oral rodent studies based on Guideline 870.3100
The CHECKED (X) parameters were examined.

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6. Urinalysis:

Urinalysis parameters were not included in this study. Urinalysis was included as part of a subchronic investigation with the test substance in rats (Report No. 201136).

7. Sacrifice and Pathology:

7a. Gross pathology:

All animals surviving to scheduled termination were sacrificed by CO_2 asphyxiation and subject to a postmortem examination, which included (1) documenting and saving all gross lesions, (2) weighing designated organs, and (3) collecting representative tissue specimens for histopathologic evaluation. Tissues were preserved (nominally in 10% buffered formalin) and selected organs were weighed and organ/body weight ratios calculated.

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	DIGESTIVE SYSTEM		CARDIOVASC./HEMAT.		NEUROLOGIC
	Bile duct (rat)	X	Aorta*	XX	Brain*+
X	Cecum*	X	Bone marrow*	X	Cerebellum
X	Colon*	XX	Heart*+	X	Cerebrum-Midbrain
X	Duodenum*	X	Lymph node, cervical*	X	Medulla/Pons
X	Esophagus*	X	Lymph node, mesenteric*	X	Eyes*
X	Gall bladder (not rat)*	XX	Spleen*+	X	Nerve, optic*
X	Ileum*	X	Thymus*+	X	Nerve, sciatic*
X	Jejunum*			X	Pituitary*
XX	Liver*+		**UROGENITAL**	X	Spinal cord, cervical*
X	Pancreas*	X	Cervix	X	Spinal cord, lumbar*
X	Rectum*	X	Clitoral gland^	X	Spinal cord, thoracic*
X	Salivary glands*	X	Epididymides*+		
X	Stomach, glandular*	XX	Kidneys*+		**OTHER**
X	Stomach, non-glandular*	X	Mammary gland*	X	Bone, femur
X	Tongue	XX	Ovary*+	X	Bone, rib/cc jct
X	Tooth	X	Preputial gland^	X	Bone, sternum
		X	Prostate*	X	Gross lesions and masses*
	RESPIRATORY	X	Seminal vesicle*	X	Harderian gland
X	Larynx*	XX	Testicle*+	X	Joint, fem/tib
XX	Lung*	X	Urinary bladder*	X	Muscle, protocol
X	Nasal structure	X	Uterus*+	X	Physical Identifier (ID chip)
X	Nasopharynx*	X	Vagina^	X	Skin, protocol*
X	Oral structure			X	Zymbal's gland^
X	Trachea*		**GLANDULAR**		
		XX	Adrenal gland*+		
		X	Exorbital/lacrimal gland^		
		X	Parathyroid*		
		X	Thyroid*		

* Recommended for 90-day oral rodent studies based on Guideline 870.3100

+ Organ weights required for rodent studies.

The CHECKED (X) tissues were collected for histological examination. The organs CHECKED (XX) were also weighed. The organs CHECKED (^) were preserved for possible micropathologic evaluation only.

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7b. Micropathology:
With the exception of the physical identifier (microchip), the vagina, and the exorbital/lacrimal, clitoral, preputial, and Zymbal glands (of which the latter 5 were taken and preserved for possible micropathologic evaluation), representative sections of the tissues were processed for all control (0 ppm) and high-dose (7,000 ppm) animals, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under the light microscope. Tissues in which compound-related effects were detected were to be examined at lower doses as necessary to establish no-observed-effect levels. Histopathological lesions were assigned a semi-quantitative ranking of the severity grade/stage from "Normal (within normal limits)" to the "most severe case (Grade 5)," as described previously.

II. Results and Discussion:

Findings:
General observations: No treatment-related clinical signs or mortalities were observed. Body weight was not affected at any dose level in either sex. No relevant effect on feed intake was determined.

Hematology: Standard hematologic endpoints were not affected.

Clinical chemistry: Selected clinical chemistry endpoints (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine, blood urea nitrogen, cholesterol and triglyceride) were not affected.

Gross necropsy, organ weights: No treatment-related macroscopic changes were noted. Absolute and relative organ weights showed no treatment-related changes.

Histopathology: No treatment-related microscopic findings were noted.

III. Conclusions: The subchronic toxicity study in mice established a NOAEL of 7,000 ppm (equal to 1,305 or 1,515 mg/kg bw/day in males or females, respectively), based on the lack of findings at any dietary level tested.

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IIA 5.3.3 Oral 90-day toxicity (dog)

Report:	**KIIA 5.3.3/01, ; 2005**
Title:	Technical Grade BYI 08330, A 90-Day Subchronic Toxicity Feeding Study in the Beagle Dog
Report No & Document No	201233 M-254183-01-1
Guidelines:	OECD 409 (SEP 1998), US-EPA-OPPTS 870.3150 (1998), JMAFF, Ref. No. 12 Nousan No. 8147, (NOV 2000). Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory). Deviations: none

Executive Summary

In a 90-day feeding study, BYI 08330, batch no.: 08045/0014, purity: 97.8 %, was administered for about 13 weeks to four male and four female Beagle dogs per group at dietary concentrations of 0, 150, 300, 1,200, and 4,000/2,500 ppm (equal to 0, 5, 9, 33 or 81 mg/kg bw/day for males and 0, 6, 10, 32 or 72 mg/kg bw/day for females, respectively).
During the first two weeks of the study, profound/precipitous declines in body weight were observed in both sexes at 4,000 ppm. After week two, the dose was reduced to 2,500 ppm and, though body weights did not return to control levels, the compound-related decline reversed sufficiently to maintain the health and well-being of the animals for the duration of the study.
Compound-related declines in circulating Thyroxine (T4) were observed in both sexes of the 1,200- and 2,500-ppm animals and of Triiodothyronine (T3) at 2,500 ppm. No changes in thyroid weight, thyroid pathology, no compensating increases in thyrotropin (TSH), or no clinical observations (e.g., neurological signs) suggestive of thyroid compromise were detected in either sex. Ultimately, based on the total response of the animals to the thyroid profile that emerged over 90 days, the compound-induced changes in circulating thyroid hormones, though significant in magnitude, were judged to be nonadverse. This conclusion was later confirmed when a similar thyroid and toxicological profile emerged in the 1-year chronic dog study.
The NOAEL of 2,500 ppm (equal to 81 or 72 mg/kg bw/day in males or females, respectively) is based on a body weight decline noted during the first two weeks of the study at 4,000 ppm. No marked toxicity was determined in the present study.
The NOEL of 300 ppm (equivalent to 9 or 10 mg/kg bw/day in males or females, respectively) is based on nonadverse declines in T4 levels at 1,200 ppm, equivalent to 33 mg/kg bw/day in males or 32 mg/kg bw/day in females.

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I. Material and methods

A. Materials:

1. Test Material:	BYI 08330
Description:	technical grade, white powder, room temperature storage
Lot/Batch No.:	08045/0014
Purity (% ai):	97.8% (5-Dec-03); 97.6% (28-Jun-04)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. if TGAI:	203313-25-1

2. Vehicle and/or positive control: corn oil and acetone

3. Test animals:	Dog, males and females	
Species:	*Canis familiaris*	
Strain:	Beagle (nulliparous and nonpregnant)	
Age/weight at study initiation:	7 - 8 months / males: 9.2 - 11.6 kg; females: 6.1 - 8.8 kg	
Source:	[redacted]	
Housing:	individually housed in stainless steel runs	
Diet:	[redacted] Canine Diet 5006-3 available for *ad libitum* consumption	
Water:	tap water provided continuously for *ad libitum* consumption	
Environmental conditions:	**Temperature:**	64 to 84°F
	Humidity:	30 to 70%
	Air changes:	Averaged at least 16 changes per hour during the dosing period*
	Photoperiod:	12 hrs of light alternating with 12 hrs of darkness
Acclimatisation:	7 days prior to release for the study	

*For a 4-week period, there were numerous cases of zero reading for air changes per hour due to an error in the setting of the microprocessor which monitored the air flow.

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B. Study design:

1. In life dates

Initiation of collection of Data (Day Animals Released for Study): December 17, 2003
Initiation of Dosing: December 22, 2003
Terminal Sacrifice: March 23 - 26, 2004

2. Animal assignment

Prior to administering the test substance, male and female animals were randomly assigned, based on weight, to the dose groups noted in Table 1. The weight variation of the animals was targeted not to exceed ±20% of the mean weight for each sex.

Dose Group (ppm)	No. Animals/ Dose/Sex	Males (mg/kg/day)	Females (mg/kg/day)
0 (control)	4	0	0
150	4	5	6
300	4	9	10
1,200	4	33	32
2,500*	4	81**	72**

* Dogs were dosed for two weeks at 4,000 ppm at which time the dose was lowered to 2,500 ppm due to excessive weight loss at 4,000 ppm.
** Dose in mg/kg/day does not include dose received at 4,000 ppm.

3. Dose selection rationale

The nominal concentrations chosen for this study were 0 (concurrent vehicle control), 150, 300, 1,200, and 2,500 ppm (4/sex/dose) of technical grade BYI 08330 mixed with dog ration. Dose levels were selected based on the results of a 28-day feeding study in the Beagle dog in which BYI 08330 was administered at doses of 0, 100, 400, 1,600, or 6,400 ppm. A marked reduction in food consumption and body weight was noted in the 6,400-ppm animals. Effects on relative organ weights were observed in 6,400-ppm animals as well as a decrease in circulating thyroxine (T4), which was noted in 400-ppm males and 6,400-ppm females.

4. Diet preparation and analysis

All feed mixtures were prepared weekly by mixing appropriate amounts of test substance with [redacted] Certified Canine Diet 5006-3 and then storing the mixture under freezer conditions until administered to the animals. Corn oil, at 1% by weight of the diet, along with acetone was used as vehicles to 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 mixed feed was taken and retained under freezer conditions until the study was complete and the study data deemed satisfactory. Prior to conducting a 4-week pilot study in the dog (Report No. 201012), the homogeneity and stability of BYI 08330 in dog ration was confirmed for dietary concentrations ranging from as low as 100 ppm to as high as 6,400 ppm in the feed.

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Results:
Based on the findings described below, the analytical data indicated that the mixing procedure was adequate and that the variance between nominal and actual dosage to the animals was acceptable.

Homogeneity analysis: Batches of ration mixed with BYI 08330 at 100 and 6,400 ppm were analyzed for homogeneity. Nine samples of ration were taken from each level and analyzed. The mean concentrations were 96.2 ppm (96.2%, CV=4.3%) for the 100 ppm test level and 6,255 ppm (97.7%, CV= 3.5%) for the 6400 ppm test level. Based on CV's of 4.3 and 3.5%, BYI 08330 mixed in dog ration at concentrations of 100 and 6400 ppm was considered homogeneously distributed.

Stability analysis: For freezer stability, samples were analyzed on day 0, 7, 14, and 28. After 28 days, there was no decline in concentration for the 100 and 6,400 ppm levels. BYI 08330 at mixed in dog ration at concentrations of 100 and 6,400 ppm was considered stable at freezer temperature for a minimum of 28 days. For room temperature stability, samples were analyzed on day 0, 1, 4, and 7. After 7 days, declines in concentration ranged from 9.8 to 2.1% for 100 and 6,400 ppm levels, respectively. BYI 08330, mixed in dog ration at concentrations of 100 and 6,400 ppm, was considered stable at room temperature for a minimum of 7 days.

Concentration analysis. During the study mixed feed from all dose groups, including control, was analyzed for stability and concentration during weeks (wks) 1 thru 7 (exceptions during wks 4 and 5) as well as wks 11 and 14. The mean concentrations for the study were 99 to 104 % of the nominal levels; %RSD values ranged from 1.38 to 4.59.

5. Statistics
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 histopathological lesion evaluations which 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.

C. Methods:

1. Observations:
1a. Cageside observations
All study animals were observed at least twice daily for clinical signs of toxicity (except once daily on weekends and holidays).

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1b. Clinical examinations

Detailed clinical observations for clinical signs of toxicity were performed on all animals at study initiation and 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. The intake of test compound in mg/kg/day was calculated for males and females using the analytical concentration of BYI 08330 in the feed and the following equation: (average food consumption per week/average body weight per week) x ppm in the feed/1,000.

4. Ophthalmoscopic examination

Following the Acclimatisation 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 & Clinical Chemistry

Clinical chemistry and a complete blood count, including differentials, were performed on all animals (pre-exposure and during weeks (wks) 4, 8, and 13). Animals were fasted overnight prior to the collection of blood, which was drawn via jugular venipuncture. The parameters evaluated are marked (x) in the lists below.

5a. Hematology

x	Hematocrit (HCT)	x	Leukocyte differential count
x	Hemoglobin (HGB)	x	Mean corpuscular hemoglobin (MCH)
x	Leukocyte count (WBC)	x	Mean corpuscular hemoglobin conc.(MCHC)
x	Erythrocyte count (RBC)	x	Mean corpuscular volume (MCV)
x	Platelet count	x	Reticulocyte count
	Blood clotting measurements		
x	(Thromboplastin time)		
	(Clotting time)		
x	(Prothrombin time)		

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5b. Clinical Chemistry

	ELECTROLYTES		OTHER
x	Calcium (calc)	x	Albumin (ALB)
x	Chloride (Cl)	x	Creatinine (Creat)
	Magnesium (Mg)	x	Urea nitrogen (Urea-N)
x	Phosphorous (Phos)	x	Total Cholesterol (Chol)
x	Potassium (K)	x	Globulins (Glob)
x	Sodium (Na)	x	Glucose (gluc)
	ENZYMES	x	Total bilirubin (T-Bili)
x	Alkaline phosphatase (ALK)	x	Total protein (TP)
	Cholinesterase (ChE)	x	Triglycerides (Trig)
x	Creatine phosphokinase (CK)		Serum protein electrophoresis
x	Lactic acid dehydrogenase (LD)	x	Uric Acid (Uric-A)
x	Alanine aminotransferase (ALT / SGPT)		
x	Aspartate aminotransferase (AST / SGOT)		
x	Gamma Glutamyl transferase (GGT)	x	T4 (T4)
	Glutamate dehydrogenase	x	T3 (T3)
		x	Thyroid Stimulating Hormone (TSH)

6. Urinalysis

Urine was collected from all animals (pre-exposure and during wks 4, 8, and 13). The parameters evaluated are marked (x) in the list below.

x	Appearance	x	Glucose (Glu)
x	Volume (UVol)	x	Ketones (Ket)
x	Specific gravity/osmolality (Sp.Gr.)	x	Bilirubin (Bil)
x	pH (pH)	x	Blood (Bld)
x	Sediment (microscopic)	x	Nitrite (Nit)
x	Protein (Pro)	x	Urobilinogen (Uro)
x	Urine Creatinine, 24 hr (Creat)	x	Leukocytes (U-Leu)

7. Sacrifice and Pathology

Animals were euthanized at the end of the study by intravenous injection of Fatal-Plus® ( ). A complete gross examination was performed on all animals. The necropsy consisted of a systematic gross examination of each animal's general physical condition, body orifices, external and internal organs and tissues. All tissues were processed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under the light microscope. Histopathologic evaluation was conducted on all protocol-required tissues from the control and high-dose animals as well as selected tissues (liver, thyroid, thymus, testicles, and epididymides) from the low-, mid-, and high-dose animals.

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Where appropriate, all findings were assigned a severity score where N = tissues within normal histological limits, 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. The mean severity was determined by dividing the sum of the individual animal severity scores by the number of tissues examined in the group. A list of the tissues collected (x) and weighed (xx) at necropsy is presented in the table below.

	DIGESTIVE SYSTEM		**CARDIOVASC./ HEMAT.**		**NEUROLOGIC**
	Tongue	x	Aorta, thoracic	xx	Brain
x	Salivary glands	xx	Heart	x	Peripheral nerve
x	Esophagus	x	Bone marrow	x	Spinal cord (3 levels)
x	Stomach	x	Lymph nodes	x	Pituitary
x	Duodenum	xx	Spleen	x	Eyes (optic nerve)
x	Jejunum	xx	Thymus		**GLANDULAR**
x	Ileum			xx	Adrenal gland
x	Cecum		**UROGENITAL**		Lacrimal gland
x	Colon	xx	Kidney	xx	Parathyroid
x	Rectum	x	Urinary bladder	xx	Thyroid
xx	Liver	x	Testes		**OTHER**
xx	Gall bladder	xx	Epididymides	x	Bone (sternum and/or femur)
x	Pancreas	x	Prostate	x	Skeletal muscle
	RESPIRATORY	xx	Ovaries	x	Skin
x	Trachea	xx	Uterus	x	All gross lesions and masses
x	Lung	x	Mammary gland		
x	Nose				
x	Pharynx				
x	Larynx				

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II. Results and Discussions:

Findings:

General observations: No treatment-related clinical signs were observed, no mortalities occurred. There was no treatment-related effect on food consumption. During the first two weeks of the study, there was a treatment-related reduction in body weight in the 4,000 ppm dose group. After week two, when the 4,000 ppm dose was reduced to 2,500 ppm, there was no treatment-related effect on body weight.

Hematology, urinalysis: No treatment-related findings.

Clinical chemistry: There was a treatment-related decrease in T_4 in the 1,200 and 2,500 ppm male and female dose groups. The findings on study day 30 for the males in the 300 ppm dose group was not considered to be treatment-related as on study days 58 and 86 the T_4 values for males were not statistically significantly below the control group. The T_4 values for females in the 150 ppm dose group were statistically significantly lower than the control group on study days 30, 58 and 86, but this finding is not considered treatment-related due to the absence of significant declines at 300 ppm. There was a slight, but statistically significant decrease in T_3 in the 2,500 ppm male and female dose groups on study days 30 and 58 which is possibly considered to be treatment-related. However, on study day 86, T_3 levels were comparable to the control mean.

Despite percentage declines in T_4 at some time points of greater than 50%, no changes in thyroid weight, thyroid pathology, no compensating increases in thyrotropin (TSH), or no clinical observations (e.g., neurological signs) suggestive of thyroid compromise were detected in either sex. Ultimately, based on the total response of the animals to the thyroid profile that emerged over 90 days, the compound-induced changes in circulating thyroid hormones, though significant in magnitude, were judged to be nonadverse. This conclusion was later confirmed when a similar thyroid and toxicological profile emerged in the 1-year chronic dog study.

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Table 5.3.3-1: Mean thyroxine (T 4) levels

Parameter		Dose Group (ppm)									
		0		150		300		1,200		2,500	
Males											
T 4	day -3	1.70		2.29		1.76		2.11		1.99	
T 4	day 30	1.87		1.60		1.34	*	0.84	*	0.71	*
T 4	day 58	1.91		1.67		1.60		1.74		0.80	
T 4	day 86	1.85		1.70		1.42		0.75	*	0.45	*
Females											
T 4	day -3	2.21		1.91		2.99		5.06	*	3.31	
T 4	day 30	2.82		1.49	*	1.94		1.03	*	0.59	*
T 4	day 58	2.82		1.94	*	2.61		1.24	*	0.94	*
T 4	day 86	2.75		1.29	*	1.50		1.34	*	1.00	*

* = $p < 0.05$

Table 5.3.3-2: Mean thyroxine (T 3) levels

Parameter		Dose Group (ppm)									
		0		150		300		1,200		2,500	
Males											
T 3	day -3	0.87		0.95		1.00		0.98		0.84	
T 3	day 30	0.76		0.77		0.78		0.63		0.49	*
T 3	day 58	0.73		0.71		0.62		0.64		0.42	*
T 3	day 86	0.97		0.98		0.97		0.90		0.79	
Females											
T 3	day -3	0.72		0.67		0.84		2.76	*	1.99	*
T 3	day 30	0.80		0.64		0.81		0.73		0.43	*
T 3	day 58	0.65		0.59		0.69		0.57		0.31	*
T 3	day 86	1.03		0.75		0.91		0.86		0.72	

* = $p < 0.05$

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Table 5.3.3-3: Mean values of affected clinical chemical parameters in blood

Parameter		Dose Group (ppm)									
		0		150		300		1,200		2,500	
Males											
TSH	day -3	0.12		0.04	*	0.08		0.18		0.10	
TSH	day 30	0.12		0.06		0.10		0.11		0.06	
TSH	day 58	0.09		0.04		0.06		0.08		0.03	
TSH	day 86	0.07		0.06		0.07		0.13		0.06	
Females											
TSH	day -3	0.12		0.09		0.17		0.16		0.21	
TSH	day 30	0.14		0.13		0.19		0.13		0.09	
TSH	day 58	0.07		0.11		0.14		0.04		0.10	
TSH	day 86	0.08		0.08		0.11		0.05		0.24	

* = $p < 0.05$

Ophthalmology: No treatment-related effects.

Gross necropsy, organ weights, histopathology: No treatment-related macroscopic changes were seen. There were no treatment-related effects on organ weights.
The statistically significant increase in relative liver weights for males in the 150, 300, and 2,500 ppm groups is considered to be incidental as there is no dose response, the magnitude of differences were slight, and there was no statistical difference in the absolute liver weights.
No treatment-related microscopic changes were seen.

III. Conclusion: The NOAEL of 2,500 ppm (equal to 81 or 72 mg/kg bw/day in males or females, respectively) in this 90-day feeding study in dogs is based on a body weight decline noted during the first two weeks of the study at 4,000 ppm. No marked toxicity was determined in the present study. Despite percentage thyroid hormone declines at some time points of greater than 50%, no changes in thyroid weight, thyroid pathology, no compensating increases in thyrotropin (TSH), or no clinical observations (e.g., neurological signs) suggestive of thyroid compromise were detected in either sex. Based on the total response of the animals to the thyroid profile that emerged over 90 days, the compound-induced changes in circulating thyroid hormones, though significant in magnitude, were judged to be nonadverse. This conclusion was later confirmed when a similar thyroid and toxicological profile emerged in the 1-year chronic dog study (see below).

The NOEL of 300 ppm (equivalent to 9 or 10 mg/kg bw/day in males or females, respectively) is based on nonadverse declines in circulating thyroxine levels at 1,200 ppm, equivalent to 33 mg/kg bw/day in males or 32 mg/kg bw/day in females.

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IIA 5.3.4 Oral 1 year toxicity (dog)

Report:	**KIIA 5.3.4/01, ; 2006**
Title:	Technical Grade BYI 08330, A Chronic Toxicity Feeding Study in the Beagle Dog
Report No &	201486
Document No	M-274969-01-1
Guidelines:	U.S. EPA Health Effects Test Guidelines, OPPTS 870.4100,OECD Guidelines for Testing of Chemicals, Section 4, Guideline 452. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a 1-year feeding study, BYI 08330, batch no.: 08045/0014, purity: 97.6 – 98.5 %, was administered for approximately one year to four male and four female Beagle dogs per group at dietary concentrations of 0, 200, 600, and 1,800 ppm (equal to 0, 6, 20, or 55 mg/kg bw/day for males and 0, 5, 19 or 48 mg/kg bw/day for females, respectively). For evaluation of T4, T3, and TSH an additional blood sampling was done during study week 22 (day 155). Special statistical analyses of T4 and T3 were done using a repeated-measures analysis of variance.

Treatment-related declines in circulating T4 were noted in males and in females at 600 ppm and at 1,800 ppm and declines in circulating T3 in males at 1,800 ppm. Micropathologically, a reduction in follicular size, possibly reflecting a reduction in the amount of colloid present in the gland, was observed in two of four males at 1,800 ppm, the highest dose tested.

The chronic NOAEL in males was 600 ppm (equal to 20 mg/kg bw/day), based on a reduced thyroid follicular size noted in two animals at 1,800 ppm (equal to 55 mg/kg bw/day).

The chronic NOAEL in females was 1,800 ppm (equal to 48 mg/kg bw/day), based on the absence of adverse compound-induced toxicological responses.

Despite thyroid hormone declines at some time points, no changes in thyroid weight, thyroid pathology, no compensating increases in thyrotropin (TSH), or no clinical observations (e.g., neurological signs) suggestive of thyroid compromise were detected in either sex. Based on the total response of the animals to the thyroid profile that emerged over one year, the isolated compound-induced changes in circulating thyroid hormones observed in this one-year study, though significant in magnitude, are judged to be nonadverse.

The overall NOEL for Beagle dogs in a chronic one year dog study with technical grade BYI 08330 was 200 ppm (equal to 5 mg/kg bw/day), based on nonadverse declines in T4 at 600 ppm, equivalent to 20 mg/kg bw/day in males and 19 mg/kg bw/day in females.

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I. Material and methods

A. Material

1. Test material: BYI 08330

Description: technical grade, white powder

Lot/Batch No.: 08045/0014

Purity (% ai)*: 97.8 (12/5/03); 97.6% (6/28/04); 98.5% (1/27/05); 98.1% (11/17/05)

Compound Stability room temperature storage

Chemical Name: 3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate

CAS No. if TGAI: Non-Stereo: 382608-10-8; CIS 203313-25-1

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2. Vehicle and/or positive control: corn oil and acetone

3. Test animals: Dog, males and females

Species: *Canis familiaris*

Strain: Beagle (nulliparous and nonpregnant)

Age/weight at study initiation: 7- 8 months / males: 6.6 - 8.5 kg & females: 5.7 - 7.9 kg

Source:

Housing: Individually housed in stainless steel cages.

Diet: Canine Diet Etts 5006-3; presented to the animals for 3-6 hours/day, beginning five days prior to initiation of treatment with the test compound and continuing throughout the study, although there were two occasions when the length of the timed feeding was not documented. Prior to this time food was available for *ad libitum* consumption, except for when the animals were fasted overnight for pre-clinical blood analyses.

Water: tap water provided continuously for *ad libitum* consumption

Environmental conditions:

Temperature: Set to be maintained at 18 to 29°C (64 to 84°F).

Humidity: Set to be maintained at 30 to 70%. There were numerous cases when the relative humidity was below 30% and above 70%. The relative humidity was below 30% during the months when the outside air was cold and dry; the HVAC unit was unable to maintain the relative humidity above 30%. When the relative humidity was above 70%, this was usually caused by flushing; also during the hot humid months the HVAC unit was unable to maintain the relative humidity below 70%.

Air changes: Averaged at least 13.00 changes per hour during the dosing period.

Photoperiod: Approximately 12 hours of light alternating with 12 hours of darkness.

Acclimatisation: 5 days prior to release for the study.

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B. Study design:

1. In life dates

Study Initiation (Day protocol was signed): May 28, 2004
Initiation of collection of Data (Day Animals Released for Study): June 1, 2004
Initiation of Dosing: June 9, 2004
Terminal Sacrifice: June 14, 2005

2. Animal assignment

The dogs were randomly assigned to dose groups, based on weight, using INSTEM DATATOX®. Weight variation of animals used were 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.

Dose Group (ppm)	No. Animals/ Dose/Sex	Males (mg/kg/day)	Females (mg/kg/day)
0 (control)	4	0	0
200	4	6	5
600	4	20	19
1,800	4	55	48

3. Dose selection rationale

The nominal concentrations chosen for this study were 0 (concurrent vehicle control) 200, 600, and 1,800 ppm (4/sex/dose) of technical grade BYI 08330 mixed with dog ration. Selection of these dose levels was based on a 90 day feeding study in the Beagle dog (Bayer Report No. 201223, with doses of 0 (concurrent vehicle control) 150, 300, 1,200, and 4,000/2,500 ppm in the diet. In this study, there was a marked reduction in the body weight for both sexes in the 4,000 ppm dose group, which was reduced to 2,500 ppm on study day 15. A dose related decrease in T_4 in the1,200 and 2,500 ppm male and female dose groups throughout the entire study, and a decrease in T_3 in the 2,500 ppm male and female dose groups on study days 30 and 58 was observed.

4. Diet preparation and analysis

All feed mixtures were prepared weekly by mixing appropriate amounts of the test substance with ███ ██████████ Canine Diet 5006-3 and then storing the mixture under freezer conditions until given to the animals. Corn oil, at 1% by weight of the diet, along with acetone was used as vehicles to 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. Prior to conducting a 4-week pilot study in the dog (Report No. 201012), the homogeneity and stability of BYI 08330 in dog ration was confirmed for concentrations of 100 ppm and 6,400 ppm.

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Results:

Based on the findings described below, the analytical data indicated that the mixing procedure was adequate and that the variance between nominal and actual dosage to the animals was acceptable.

Homogeneity analysis:

Batches of ration mixed with BYI 08330 at 100 and 6,400 ppm were analyzed for homogeneity. Nine samples of ration were taken from each level and analyzed. The mean concentrations were 96.2 ppm (96.2%, CV = 4.3%) for the 100 ppm test level and 6,255 ppm (97.7%, CV = 3.5%) for the 6,400 ppm test level. Based on CV's of 4.3 and 3.5%, BYI 08330 mixed in dog ration at concentrations of 100 and 6,400 ppm was considered homogeneously distributed.

Stability analysis:

For freezer stability, samples were analyzed on day 0, 7, 14, and 28. After 28 days, there was no decline in concentration for the 100 and 6,400 ppm levels. BYI 08330 at concentrations of 100 and 6,400 ppm was considered stable at freezer temperature in dog ration for a minimum of 28 days. For room temperature stability, samples were analyzed on day 0, 1, 4, and 7. After 7 days, declines in concentration ranged from 9.8 to 2.1% for 100 and 6,400 ppm levels, respectively. BYI 08330, mixed in dog ration at concentrations of 100 and 6,400 ppm, was considered stable at room temperature for a minimum of 7 days .

Concentration analysis:

The concentration of the active ingredient in the feed was verified for the first three weeks of the study and then monthly thereafter. The mean concentrations for the study were 94 to 100% of the nominal levels; %RSD values ranged from 4.58 to 6.14.

5. Statistics

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 histopathological lesion evaluations which 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.

A complementary statistical analysis of T_4 and T_3 values was conducted by , expert in statistics and epidemiology, at , France. The T_4 and T_3 values were log-transformed and analyzed with a repeated-measures analysis of variance (ANOVA, type III sum of squares) including terms for treatment, time and interaction of treatment and time. Repeated measurements analysis was performed assuming an unstructured covariance matrix.

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When the ANOVA indicated a statistically significant overall treatment or interaction effect ($p \leq 0.05$), multiple t-tests were applied using the ANOVA's least-squares (LS) means and corresponding standard errors. Statistical significances were defined at the 5% and 1% levels of significance.

C. Methods:

1. Observations:

1a. Cageside observations

All study animals were observed at least twice daily for clinical signs of toxicity (except once daily on weekends and holidays).

1b. Clinical examinations

Detailed clinical observations for clinical signs of toxicity were performed on all animals at study initiation and 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. The intake of test compound in mg/kg/day was calculated for males and females using the analytical concentration of BYI 08330 in the feed and the following equation: (average food consumption per week/average body weight per week) x ppm in the feed/1,000.

4. Ophthalmoscopic examination

Following the Acclimatisation 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 & clinical chemistry

Clinical chemistry and a complete blood count, including differentials, were performed on all animals (preexposure and during study weeks 13, 25, 38, and 51). During study week 22, blood was collected from all animals for evaluation of T_4, T_3, and TSH. Animals were fasted overnight prior to the collection of blood (except during week 22), which was drawn via jugular venipuncture. The parameters evaluated are marked (x) in the lists below.

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5a. Hematology

x	Hematocrit (HCT)*	x	Leukocyte differential count*
x	Hemoglobin (HGB)*	x	Mean corpuscular hemoglobin (MCH)*
x	Leukocyte count (WBC)*	x	Mean corpuscular hemoglobin conc.(MCHC)*
x	Erythrocyte count (RBC)*	x	Mean corpuscular volume (MCV)*
x	Platelet count*	x	Reticulocyte count
	Blood clotting measurements*	x	Blood cell morphology
x	(Thromboplastin time)	x	Red Blood Cell Distribution Width (RDW)
	(Clotting time)	x	Hemoglobin Distribution Width (HDW)
x	(Prothrombin time)		

*Recommended for chronic studies based on Guideline 870.4100

5b. Clinical Chemistry

	ELECTROLYTES		**OTHER**
x	Calcium (calc)*	x	Albumin (ALB)*
x	Chloride (Cl)*	x	Creatinine (Creat)*
	Magnesium (Mg)*	x	Urea nitrogen (Urea-N)*
x	Phosphorous (Phos)*	x	Total Cholesterol (Chol)*
x	Potassium (K)*	x	Globulins (Glob)
x	Sodium (Na)*	x	Glucose (gluc)*
	ENZYMES (more than 2 hepatic enzymes)*	x	Total bilirubin (T-Bili)
x	Alkaline phosphatase (ALK)*	x	Total protein (TP)*
	Cholinesterase (ChE)	x	Triglycerides (Trig)
x	Creatine phosphokinase (CK)		Serum protein electrophoresis
x	Lactic acid dehydrogenase (LD)	x	Uric Acid (Uric-A)
x	Alanine aminotransferase (ALT/SGPT)*		Bile Acids
x	Aspartate aminotransferase (AST/SGOT)*	x	A/G ratio (A\G)
x	Gamma Glutamyl transferase (GGT)*	x	T4 (T4)
	Glutamate dehydrogenase	x	T3 (T3)
		x	Thyroid Stimulating Hormone (TSH)

*Recommended for chronic studies based on Guideline 870.4100

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6. Urinalysis

Urine was collected from all animals (pre-exposure and during wks 4, 8, and 13). The parameters evaluated are marked with (x) in the list below.

x	Appearance*	x	Glucose (Glu)*
x	Volume (UVol)*	x	Ketones (Ket)
x	Specific gravity / osmolality (Sp.Gr.)*	x	Bilirubin (Bil)
x	pH (pH)*	x	Blood (Bld)*
x	Sediment (microscopic)	x	Nitrite (Nit)
x	Protein (Pro)*	x	Urobilinogen (Uro)
x	Urine Creatinine (24 hour) (Creat)	x	Leucocytes (U-Leu)

*Recommended for chronic studies based on Guideline 870.4100

7. Sacrifice and pathology

Animals were euthanized at the end of the study by intravenous injection of Fatal-Plus® ( ). A complete gross examination was performed on all animals. The necropsy consisted of a systematic gross examination of each animal's general physical condition, body orifices, external and internal organs and tissues. A list of the tissues collected and weighed at necropsy is presented in the table below. All tissues were processed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under the light microscope. Histopathologic evaluation was conducted on all protocol-required tissues from the control and high-dose group animals as well as epididymides, lungs, and thyroids from the low- and mid-dose group animals. Where appropriate, all findings were assigned a severity score where N = tissues within normal histological limits, 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. The mean severity was determined by dividing the sum of the individual animal severity scores by the number of tissues examined in the group.

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Tissues Collected at Necropsy, Organs Weighed, and Tissues Examined Microscopically

	DIGESTIVE SYSTEM		**CARDIOVASC./HEMAT**		**NEUROLOGIC**
X	Cecum*	X	Aorta*	X	Brain*+#
X	Colon*	X	Bone marrow*	X	Cerebellum
X	Duodenum*	X	Heart*+#	X	Cerebrum-Midbrain
X	Esophagus*	X	Lymph node, mesenteric*	X	Medulla/Pons
X	Gall bladder*	X	Lymph node,	X	Eyes*
X	Ileum*	X	Spleen#	X	Nerve, optic*
X	Jejunum*	X	Thymus#	X	Nerve, sciatic
X	Liver*+#			X	Pituitary#
X	Pancreas*		**UROGENITAL**	X	Spinal cord, cervical*
X	Rectum*	X	Cervix	X	Spinal cord, thoracic*
X	Salivary glands*		Clitoral gland	X	Spinal cord, lumbar*
X	Stomach	X	Epididymides*+#		
		X	Fallopian tube (oviduct)		**OTHER**
		X	Kidneys*+#		Bone, femur
		X	Mammary gland*	X	Bone marrow
		X	Ovary*#	X	Bone, rib/cc jct
		X	Prostate*	X	Bone, sternum
	RESPIRATORY	X	Testicle*+#	X	Gross lesions*
X	Larynx	X	Ureter		Joint, fem/tib
X	Lung*++	X	Urinary bladder*	X	Muscle, protocol
	Nasal structure	X	Uterus*#	X	Physical Identifier@
X	Nasopharynx*	X	Vagina@	X	Skin, protocol*
	Oral structure				
X	Trachea*		**GLANDULAR**		
		X	Adrenal gland*+#		
			Exorbital/lacrimal gland		
		X	Thyroid (with parathyroid)*		

*Required for chronic studies based on Guideline 870.4100.
+Organ weight required in chronic studies.
++Organ weight required if inhalation route.
#Organ weighed.
@No histopathology performed

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II. Results and Discussions:

Findings:

General observations: No treatment-related clinical signs were observed, no mortalities occurred. There was no compound-related effect on food consumption, body weights were not affected.

Hematology, urinalysis: No treatment-related findings.

Clinical chemistry: Thyroxine (T4) was statistically decreased in males at 1,800 ppm for all post-treatment time points and at 600 ppm at termination of study (day 357). In females, T4 was statistically decreased for most time points at 600 and 1,800 ppm. Triiodothyronine (T3) decreases were less pronounced and occurred in males at 1,800 ppm for all time points. Decreases in T4 and T3 levels at these dose levels were considered to be compound-related.

The statistical significant T4 decrease in females on day 357 at 200 ppm was considered a statistical aberration due to the high T4 mean in controls. The statistical significant T3 decrease in males on day 180 at 200 ppm and 600 ppm was considered a statistical aberration due to the high T3 mean in controls. This is also supported by the fact, that T3 levels were comparable to control levels at subsequent time points. Statistical declines in T3 were not noted in females at any dietary level tested.

However, despite declines in thyroid hormones at some time points, no changes in thyroid weight, thyroid pathology, no compensating increases in thyrotropin (TSH), or no clinical observations (e.g., neurological signs) suggestive of thyroid compromise were detected in either sex. Based on the total response of the animals to the thyroid profile that emerged over one year, the isolated compound-induced changes in circulating thyroid hormones observed in this one-year study, though significant in magnitude, are judged to be nonadverse.
Other clinical chemistry parameters were not affected by treatment with BYI 08330.

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Table 5.3.4-1: Mean thyroxine (T 4) levels

Parameter		Dose Group (ppm)								Historical range	
		0		200		600		1,800		Low	High
Males											
T 4	day -5	2.14		2.20		2.33		2.74		1.2	4.1
T 4	day 96	2.35		0.99		0.89		0.53	*	0.9	3.9
T 4	day 155	2.17		1.30		1.14		1.01	*	n.a.	n.a.
T 4	day 180	2.13		0.87		0.70		0.48	*	0.7	3.5
T 4	day 271	1.86		0.94		0.78		0.33	**	0.9	3.0
T 4	day 357	1.84		1.16		1.02	*	0.58	**	0.6	3.3
Females											
T 4	day -5	2.44		2.57		2.63		2.37		0.6	4.2
T 4	day 96	1.83		1.19		0.93	*	0.69	**	0.7	3.6
T 4	day 155	2.56		1.76		1.24	**	1.12	**	n.a.	n.a.
T 4	day 180	2.18		1.38		1.32		0.51	**	0.6	4.4
T 4	day 271	1.80		1.23		0.90	*	0.58	**	0.9	4.0
T 4	day 357	2.88		1.79	*①	1.11	**	0.85	**	0.9	4.6

* =p < 0.05; ** =p < 0.01 (repeated measures ANOVA and t-test using ANOVA's least-square means

① statistical aberration due to the high T4 in controls; n.a. = not available

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Table 5.3.4-2: Mean triiodothyronine (T 3) levels

Parameter		Dose Group (ppm)								Historical range	
		0		200		600		1,800		Low	High
Males											
T 3	day -5	0.89		0.87		0.91		0.95		0.4	1.2
T 3	day 96	0.79		0.61		0.68		0.48	**	0.4	1.3
T 3	day 155	0.71		0.60		0.62		0.52	*	n.a.	n.a.
T 3	day 180	0.82		0.64	**①	0.61	**①	0.52	**	0.6	1.4
T 3	day 271	0.64		0.49		0.50		0.43	*	0.5	1.2
T 3	day 357	0.65		0.57		0.67		0.46	**	0.6	2.3
Females											
T 3	day -5	0.80		0.80		0.78		0.87		0.3	1.3
T 3	day 96	0.70		0.73		0.69		0.62		0.5	1.2
T 3	day 155	0.69		0.62		0.66		0.59		n.a.	n.a.
T 3	day 180	0.85		0.75		0.82		0.61		0.4	1.4
T 3	day 271	0.61		0.58		0.63		0.50		0.4	1.3
T 3	day 357	0.73		0.62		0.63		0.61		0.5	1.1

* =p < 0.05; ** =p < 0.01 (repeated measures ANOVA and t-test using ANOVA's least-square means

① statistical aberration due to the relatively high T3 control mean; n.a. = not available

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Table 5.3.4-3: Mean thyrotropin (TSH) levels

Parameter		Dose Group (ppm)				Historical range	
		0	200	600	1,800	Low	High
Males							
TSH	day -5	0.18	0.33	0.17	0.22	0.02	0.54
TSH	day 96	0.16	0.17	0.11	0.11	0.07	1.10
TSH	day 155	0.17	0.33	0.15	0.12	n.a.	n.a.
TSH	day 180	0.16	0.21	0.13	0.19	0.06	0.41
TSH	day 271	0.11	0.16	0.11	0.10	0.07	0.39
TSH	day 357	0.16	0.18	0.11	0.06	0.04	0.42
Females							
TSH	day -5	0.13	0.17	0.11	0.16	0.04	0.41
TSH	day 96	0.11	0.12	0.08	0.06	0.03	0.74
TSH	day 155	0.12	0.13	0.10	0.09	n.a.	n.a.
TSH	day 180	0.21	0.21	0.17	0.07	0.06	0.49
TSH	day 271	0.11	0.12	0.09	0.09	0.04	0.39
TSH	day 357	0.14	0.10	0.08	0.06	0.07	0.46

* =p < 0.05; ** =p < 0.01 (repeated measures ANOVA and t-test using ANOVA's least-square means
n.a. = not available

Ophthalmology: No treatment-related effects.

Gross necropsy, organ weights: No treatment-related macroscopic changes were seen. Organ weights were not affected by treatment. Mean thyroid absolute and relative organ weights and other organ weights were not statistically different from controls.

Histopathology: Compound-related morphological changes were limited to male thyroids at the 1,800 ppm dose level. Two 1,800 ppm males had a slight reduction in the size of the peripheral thyroid follicles. Two additional step-sections from the blocks were also evaluated for all control and 1,800 ppm males and females. None of these additional evaluations changed the follicular morphology observed in the original slides.

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III. Conclusion:

The chronic NOAEL in males was established at 600 ppm (equal to 20 mg/kg bw/day), based on a reduced thyroid follicular size noted in two animals at 1,800 ppm (equal to 55 mg/kg bw/day).
The chronic NOAEL in females was established at 1,800 ppm (equal to 48 mg/kg bw/day), based on the absence of adverse compound-induced toxicological responses.
Despite thyroid hormone declines at some time points, no changes in thyroid weight, thyroid pathology, no compensating increases in thyrotropin (TSH), or no clinical observations (e.g., neurological signs) suggestive of thyroid compromise were detected in either sex. Based on the total response of the animals to the thyroid profile that emerged over one year, the isolated compound-induced changes in circulating thyroid hormones observed in this one-year study, though significant in magnitude, were judged to be nonadverse.
Based on nonadverse declines in T4 at 600 ppm (equivalent to 20 mg/kg bw/day in males and 19 mg/kg bw/day in females), the overall NOEL for beagle dogs in a chronic one year dog study with technical grade BYI 08330 was 200 ppm (equal to 5 mg/kg bw/day).

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IIA 5.3.5 28-day inhalation toxicity (rodents)

Testing for short-term (28 days) inhalation toxicity is not considered necessary because in an acute inhalation study BYI 08330 was shown to be of low toxicity (see IIA 5.2.3). Furthermore, BYI 08330 has a vapour pressure of 5.6×10^{-9} Pa at 20°C (see IIA, 2.3.1) which is several orders of magnitude below the level of 1×10^{-2} Pa at which Directive 94/79 EC recommends to consider to conduct a short-term inhalation study.

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IIA 5.3.6 90-day inhalation toxicity (rodents)

Not required.

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IIA 5.3.7 Percutaneous 28-day toxicity (rodents)

Report:	**KIIA 5.3.7/01, .; 2006**
Title:	Technical Grade BYI 08330, A Subacute Dermal Toxicity Study in the Rat with BYI 08330
Report No & Document No	201505 M-275227-01-1
Guidelines:	U.S. EPA-OPPTS Guideline No. 870.3200. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a 28-day dermal toxicity study, BYI 08330, batch no. 08045/0014, purity: 97.6 % to 98.5%, was administered to groups of 10 male and 10 female Wistar Hanover rats of the strain Crl:WI[G1x/BRL/Han]IGS BR at levels of 0 (control group), 100, 300, and 1,000 mg/kg by dermal application. The doses were administered for a minimum of six hours/day for five consecutive days/week for four weeks. Analytical determinations of stability and homogeneity were not performed because the test substance was applied undiluted and only moistened with water immediately before application.
No treatment-related effects were observed in either males or females at any dose level.

I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description: technical grade, white powder, room temp storage

Lot/Batch No.: 08045/0014

Purity (ai%): 97.6% (28-Jun-04); 98.5% (27-Jan-05); 98.1% (17-Nov-05)

Chemical Name: 3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate

CAS No. if TGAI: Non-Stereo: 382608-10-8; CIS 203313-25-1

---

2. Vehicle and/or positive control: distilled water

3. Test animals:

Species: Rat

Strain: Wistar Hanover CRL:WI [GLX/BRL/HAN]IGS BR (nulliparous and nonpregnant)

Age/weight at study initiation: 7-9 weeks/males: 173- 302 g & females: 149-220 g

Source:

Housing: Individually housed in stainless steel wire mesh cages

Diet: Certified Rodent Chow 5002 available for *ad libitum* consumption

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Water:	Tap water (Kansas City Missouri municipal water, dispensed by automatic watering system) was available for *ad libitum* consumption.	
Environmental conditions:	**Temperature:**	18 to 26°C
	Humidity:	30 to 70%
	Air changes:	Averaged at least 11/hr during the dosing period.
	Photoperiod:	12 hrs of light alternating with 12 hrs of darkness.
Acclimatisation:	4 or 10 days prior to release for the study	

B. Study design:

1. In life dates:
Study Initiation: January 6, 2005
Initiation of Dosing: January 10, 2005
Necropsy: February 7 and 8, 2005

2. Animal assignment, test groups and study duration:
Rats were randomly assigned to dose groups, based on weight, using INSTEM DATATOX®. Four dose groups, three treated and one concurrent control group were dosed dermally with 0 (control group), 100, 300, and 1,000 mg/kg of BYI 08330 for 28 or 29 days. Each treatment group consisted of ten males and ten females. The doses were based on each animal's body weight on days 0, 7, 14, 21.

3. Dose selection rationale: A limit dose of 1,000 mg/kg/day was used in the study.

4. Preparation and treatment of animal skin: On study day -3 (i.e., the Friday prior to dosing), the hair was removed from the dorsal and lateral areas of the trunk of each rat using electric clippers (Note: Rat YC1105 was not shaved until study day 0, due to an oversite on study day -3). During the dosing period, the animals were shaved when necessary due to hair growth. The test material was weighed out for each animal and applied to a large commercially available adhesive bandage (2 in. x 4 in. or 4.5 in.), and moistened with 1 ml of deionized water. The adhesive bandage was placed on the shaved skin of the rat and the torso of the animal was then wrapped with porous medical tape to assure that the bandage remained on the animal during the dosing interval. The same procedure was performed for control animals with the exception that only the bandage, moistened with 1 ml of deionized water, was applied to the dose site. The test substance was held in contact with the skin for a minimum of six hrs/day for five consecutive days/week for four weeks. Each day the bandage and tape 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: 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 were analyzed by Bartlett's test for homogeneity. If the data was homogeneous, an ANOVA was performed followed by Dunnett'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.

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Frequency data that were examined statistically were evaluated using the Chi-Square test. 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.

C. Methods:

1. Observations:

1a. Cageside Observations

All animals were observed at least twice daily (AM and PM) during the working week and once on the weekends.

1b. Clinical Examinations

During the week, clinical examinations were conducted twice a day during the dosing and unwrapping of the animals.

1c. Neurological Evaluations

Neurological evaluations are available from other studies since they were not performed in this study.

2. Body weight

The weight of each animal was determined on study days 0, 7, 14, 21, and 28. A terminal body weight was obtained on animals that were found dead or sacrificed *in extremis*.

3. Food consumption

Food consumption was not measured in the study.

4. Ophthalmoscopic examination

Eyes were examined on all animals pretreatment and during study week (wk) 4. Animals with pretreatment abnormalities were excluded from the study.

5. Hematology & Clinical Chemistry

During the 3rd and 4th week of the study, blood was collected from the orbital sinus of all rats under isoflurane anesthesia. During study wk 3, the blood was analyzed for standard serum chemistry and hematological parameters; during wk 4 the blood was analyzed for prothrombin time and thromboplastin time. The animals were fasted overnight prior to collecting blood during wk 3. The parameters evaluated are checked with (x) in the lists below:

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5a. Hematology

x	Hematocrit (HCT)	x	Leukocyte differential count
x	Hemoglobin (HGB)	x	Mean corpuscular hemoglobin (MCH)
x	Leukocyte count (WBC)	x	Mean corpuscular hemoglobin conc.(MCHC)
x	Erythrocyte count (RBC)	x	Mean corpuscular volume (MCV)
x	Platelet count	x	Reticulocyte count
	Blood clotting measurements:	x	Erythrocyte morphology
x	(Activated Partial Thromboplastin time)	x	Red blood cell distribution width (RDW)
	(Clotting time)	x	Hemoglobin distribution width (HDW)
x	(Prothrombin time)		

5b. Clinical Chemistry

	ELECTROLYTES		**OTHER**
x	Calcium (calc)	x	Albumin (ALB)
x	Chloride (Cl)	x	Cholesterol, total (Chol)
	Magnesium (Mg)	x	Creatinine (Creat)
x	Phosphorous (Phos)	x	Globulins (Glob)
x	Potassium (K)	x	Glucose (Gluc)
x	Sodium (Na)	x	Total bilirubin (T-Bili)
	ENZYMES	x	Total protein (T-Prot)
x	Alkaline phosphatase (ALK)	x	Triglycerides (Trig)
	Cholinesterase (ChE)	x	Urin Acid (Uric-A)
x	Creatine phosphokinase (CK)	x	A/G ratio (A/G)
x	Lactic acid dehydrogenase (LD)	x	Urea nitrogen (Urea-N)
x	Alanine aminotransferase (ALT / SGPT)		Bile Acids
x	Aspartate aminotransferase (AST / SGOT)		Serum protein electrophoresis
x	Gamma Glutamyl transferase (GGT)		
	Glutamate dehydrogenase		

6. Urinalysis

Urinalysis was not conducted in the study.

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7. Sacrifice and Pathology

A complete gross examination was performed on all animals. The necropsy consisted of a systematic gross examination of each animal's general physical condition, body orifices, external and internal organs and tissues. All tissues collected were processed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination (initially control and high-dose then proceeding to lower doses as necessary) under the light microscope. A list of the tissues collected (x) and weighed (xx) at necropsy is presented in the table below.

	DIGESTIVE SYSTEM		**CARDIOVAS. / HEMAT.**		**NEUROLOGIC**
	Bile Duct (rat)	x	Aorta	xx	Brain
x	Cecum	x	Bone Marrow	x	Cerebellum
x	Colon	xx	Heart	x	Cerebrum-Midbrain
x	Duodenum	x	Lymph node, cervical*	x	Medulla/Pons
x	Esophagus	x	Lymph node, mesenteric*	x	Eyes
	Gall Bladder (not rat)	xx	Spleen	x	Nerve, optic
x	Ileum	xx	Thymus	x	Nerve , sciatic
x	Jejunum		**UROGENITAL**	x	Pituitary
xx	Liver	x	Cervix*	x	Spinal Cord, cervical
x	Pancreas	x	Clitoral Gland*	x	Spinal Cord, thoracic
x	Rectum	xx	Epididymides	x	Spinal Cord, lumbar
x	Salivary Glands	xx	Kidney		**OTHER**
x	Stomach, glandular	x	Mammary Gland		Bone, femur
x	Stomach, non-glandular	xx	Ovary		Bone, rib/cc jct
x	Tongue*	x	Preputial Gland*	x	Bone, sternum
x	Tooth*	x	Prostate	x	Gross lesions
	RESPIRATORY	x	Seminal Vesicle	x	Harderian Gland*
x	Larynx	xx	Testicle	x	Joint, fem/tib*
x	Lung	x	Urinary Bladder	x	Muscle, protocol*
x	Nasal structure	xx	Uterus	x	Physical identifier*
x	Nasopharynx	x	Vagina*	x	Skin (treated and non-treated)
x	Oral structure		**GLANDULAR**	x	Zymbal's Gland*
x	Trachea	xx	Adrenal		
		x	Exorbital/Lacrimal Gland*		
		x	Thyroid (w/parathyroid)		

*No histopathology performed

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II. Results and Discussions:

Findings:

General observations: One female at 100 mg/kg bw/day died on study day 22 due to the anesthesia used when collecting blood. This early death was considered to be incidental and not treatment-related. All other animals survived until the scheduled termination of the study. No effects on body weight development, food consumption, clinical signs, or local skin reactions were observed.

Ophthalmology: No treatment-related effects.

Hematology: No treatment-related changes.

Clinical chemistry: No treatment-related changes.

Gross necropsy, organ weights, histopathology: No treatment-related macroscopic changes were seen. Organ weights were not affected by compound administration. There were no treatment-related microscopic observations.

III. Conclusion: The NOAEL of 1,000 mg/kg in males and females is based on the absence of effects at the highest dose tested.

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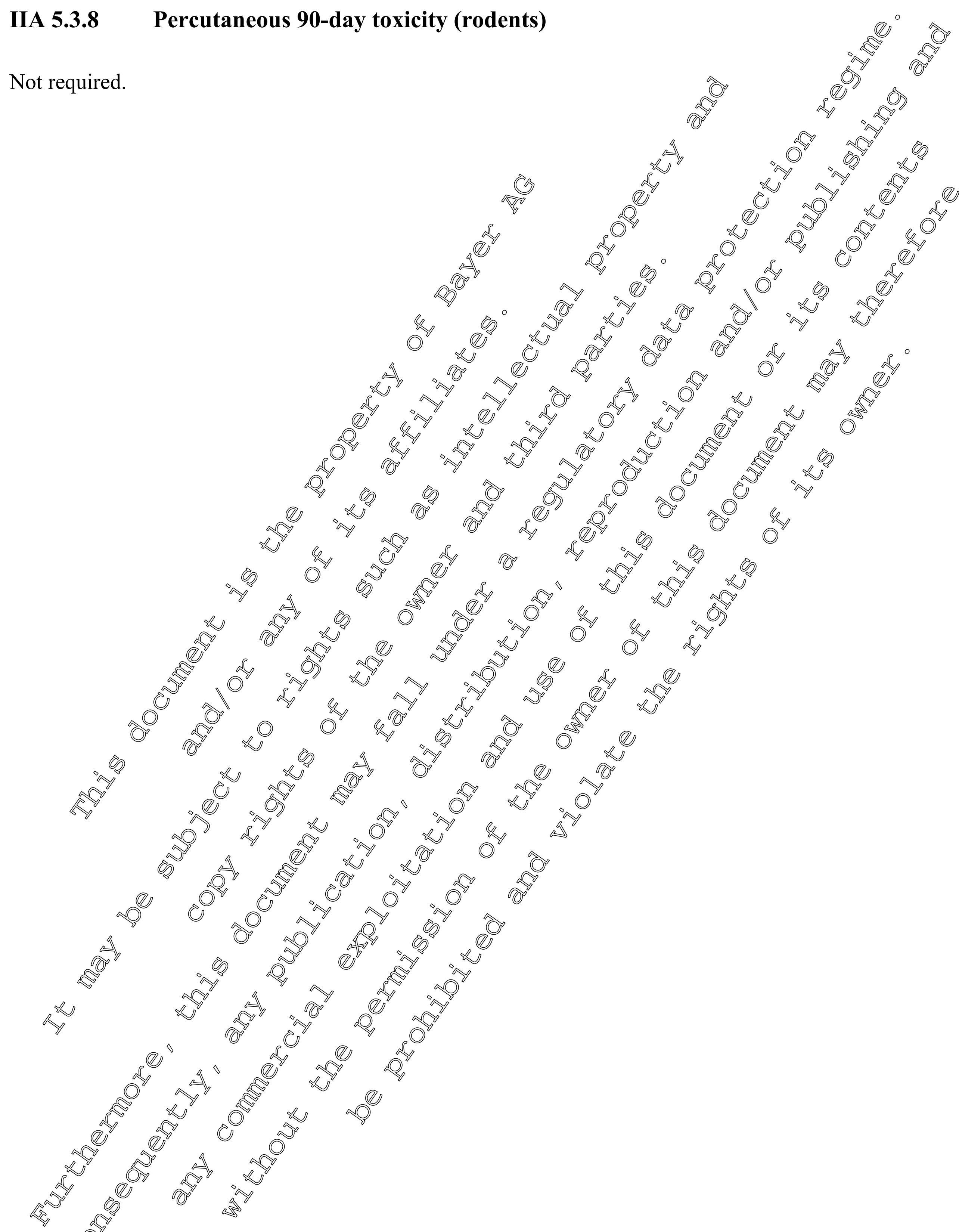
IIA 5.3.8 Percutaneous 90-day toxicity (rodents)

Not required.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IIA 5.4 Genotoxicity

Summary of genotoxicity/mutagenicity tests with BYI 08330

Test System	Concentration/Dose	Purity [%]	Results	Report No / Document No
Ames test (TA 100, TA 1535, TA 98, TA 1537 and TA 102)	+/-S9 mix: 16 – 5,000 µg/plate	93.5-96.5	negative	AT00056 / M-065358-01-2
Ames test (TA 100, TA 1535, TA 98, TA 1537 and TA 102)	+/-S9 mix: 16 – 5,000 µg/plate	95.2	negative	AT03070 / M-272000-01-2
CYT/V79 test	-S9 mix: up to 50 µg/mL +S9 mix: up to 80 µg/mL	93.5-96.5	weakly positive	AT00055 / M-065342-01-2
CYT/V79 screen	-S9 mix: up to 110 µg/mL +S9 mix: up to 120 µg/mL	98.6	negative	AT00194 / M-075136-01-2
V79/HPRT test	-S9 mix: up to 80 µg/mL +S9 mix: up to 140 µg/mL	93.5-96.5	negative	AT00137 + Amendment AT00137A / M-072857-02-2
Micronucleus test in mice	125, 250, 500 mg/kg bw	93.5-96.5	negative	AT00048 / M-065314-01-2
Bone marrow chromosome aberration test in mice	125, 250, 500 mg/kg bw	92.7-93.5	negative	AR00070 / M-084116-01-2
Unscheduled D N A synthesis test in rat liver	1,000, 2,000 mg/kg bw	92.7-93.5	negative	AT00526 M-116087-01-2

In order to support an assessment of the toxicological equivalence of different tox batches (i.e. the batch with the proposed technical specification for which approval is sought and an earlier batch which was used for genotoxicity testing), the Salmonella/Microsome test was repeated with the batch containing an impurity profile covering the proposed technical specification (Report No. AT03070).

With one exception, both point-mutagenic and chromosome aberrational (both *in vivo* and *in vitro*) testing with BYI 08330 and its metabolites were negative. A weak positive finding was noted in a single *in vitro* chromosome aberration test at the highest concentration which was assumed to be due to a secondary non-genotoxic mechanism. The test was repeated using a later, more purified, batch of test material. Negative findings in both the follow-up study as well as three subsequent *in vivo* chromosome aberration studies (using the test material employed in the 1st *in vitro* chromosome aberration test) do not suggest a mutagenic/genetic toxicity concern for BYI 08330 in the case of human exposure.

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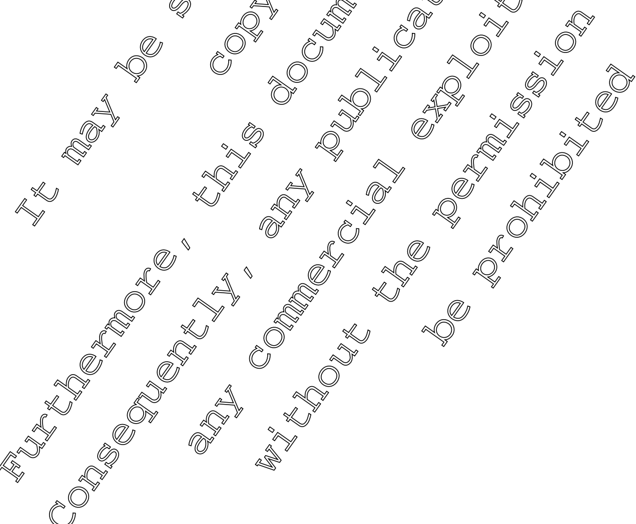
IIA 5.4.1 *In vitro* genotoxicity - Bacterial assay for gene mutation

Report:	**KIIA 5.4.1/01, 2002**
Title:	BYI 08330 – Salmonella/microsome test plate incorporation and preincubation method
Report No &	AT00056
Document No	M-065358-01-2
Guidelines:	OECD Guideline No. 471, EEC B.13/14, US EPA 712-C-98-247 OPPTS 870.5100; Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a reverse gene mutation assay in bacteria, histidine-auxotrophic Salmonella typhimurium LT2 mutant strains TA 100, TA 1535, TA 98, TA 1537 and TA 102 were exposed to BYI 08330, purity 96.5% to 93.5 %, batch no. NLL6425-9, using dimethylsulfoxide (DMSO) as solvent. The Salmonella/microsome plate incorporation test was initially investigated for point mutagenic effects at doses of 16, 50, 158, 500, 1,581 and 5,000 µg/plate on the five Salmonella strains with and without S9 mix. The independent repeat was performed as preincubation for 20 minutes at 37°C.
Doses up to and including 158 µg per plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At higher doses, the substance had a strong, strain-specific bacteriotoxic effect, so that this range could only be used up to and including 1581 ug per plate for assessment purposes.
Evidence of mutagenic activity of BYI 08330 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed.
The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant colonies compared to the corresponding negative controls.

Therefore, BYI 08330 was considered to be non-mutagenic without and with S9 mix in the plate incorporation as well as in the preincubation modification of the Salmonella/ microsome test.



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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	technical grade, white powder
Lot/Batch No.:	NLL6425-9
Purity (%ai):	96.5% (11-Dec-01); 93.5% (21-Jun-02)
Compound Stability	room temperature storage
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of	203313-25-1
Solvent Used:	DMSO

2. Control Materials:

Negative:	N/A	
Solvent (final conc'n):	DMSO, (0.1 ml/plate)	
Positive:	Nonactivation:	
	Sodium azide	10 µg/plate TA1535
	Nitrofurantoin	0.2 µg/plate TA 100
	4-Nitro-1, 2- phenylene diamine	0.5 µg/plate TA 98
	Mitomycin C	0.2 µg/plate TA 102
	4-Nitro-1, 2- phenylene diamine	10 µg/plate TA1537
	Cumene hydroperoxide	50 µg/plate TA 102
	Activation: 2-Aminoanthracene 3 µg/plate, all strains	

3. Activation: S9 derived from

x	induced	x	Aroclor 1254	x	Rat	x	Liver
	non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other		Other		

Describe S9 mix composition:

Cofactor solution: $MgCl_2 \times 6 H_2O$**:** 162.6 mg 70 mls, S9 fraction, 10 ml, KCl 0.15 M, 20 ml

KCl: 246.0 mg

Glucose-6-phospate, disodium salt: 179.1 mg

NADP, disodium salt: 315.0 mg

Phosphate buffer: 100.0 mM

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4. Test organisms: *S. typhimurium* strains

	TA97	x	TA98	x	TA100	x	TA102	TA104
x	TA1535	x	TA1537		TA1538		list any others	

Properly maintained?	x	Yes	No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	x	Yes	No

5. Test compound concentrations used:

Nonactivated conditions:
0, 16, 50, 158, 500, 1581, 5000 ug/plate, all strains, 3 replicates/treatment, 2 trials
Activated conditions:
0, 16, 50, 158, 500, 1581, 5000 ug/plate, all strains, 3 replicates/treatment, 2 trials

B. Test performance

1. Type of *Salmonella* assay:
_x_ standard plate test
_x_ pre-incubation (20 minutes)
__ "Prival" modification (*i.e. azo-reduction method*)
__ spot test
__ other

2. Protocol:
In the first trial, a strict plate incorporation method was employed (Ames *et al.* (1973) *Proc. Nat. Acad. Sci.* 70: 2281-2285, Ames *et al.* (1975) *Mutation Res.* 31: 347-364). For the mutant colony count, each treatment level was plated in triplicate. The negative control consisted of the solvent absent the test material. In the plate incorporation procedure, 0.1 ml of the solvent with or w/o the test material, 0.1 ml of the bacterial inoculum, 0.5 ml of the S9 fraction or buffer, and 2.0 ml of soft agar were warmed to 45°C in a water bath for 30 seconds, mixed and poured onto a petri dish. The incubation proceeded for 48 hours at 37 °C. The number of mutant colonies was then recorded.
In the second trial, 0.1 ml of the solvent with or w/o the test material, 0.1 ml of the bacterial inoculum, and 0.5 ml of the S9 fraction or buffer were pre-incubated for 20 minutes at 37 °C, the agar was added and mixed, followed by 48 hours of incubation at 37 °C. Likewise, the number of mutant colonies was recorded. The cytotoxicity of the test material was assessed in three ways. 1). An appraisal of the background growth on the incubation plates was assessed. 2). If there was a marked and dose-dependent reduction on the mutant count, that was considered to be a measure of toxicity. 3). Total bacterial counts were taken by incubating two plates per treatment level in the presence of histidine and incubating for 48 hours at 37 °C. A reduction in the titer in comparison to the negative control was considered to be an indication of toxicity.

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3. Statistical Analysis: No statistical analysis was performed on the study data.

4. Evaluation Criteria:

Acceptance of an assay required that:

1) The negative controls had to be within the expected range as defined by published data or the laboratories' own historical data.
2) The positive controls had to demonstrate a mutagenic effect.
3) Titer determinations had to demonstrate sufficient bacterial density in the suspension.

In order to achievea positive result, a reproducible and dose-related increase in mutant counts for at least one strain must occur. For TA1535, TA 100 and TA 98, the increase should be at least 2-fold that of the negative controls. For TA1537, there should be a 3-fold increase. For TA102, the increase should be at least 150 colonies over that counted for the negative control.

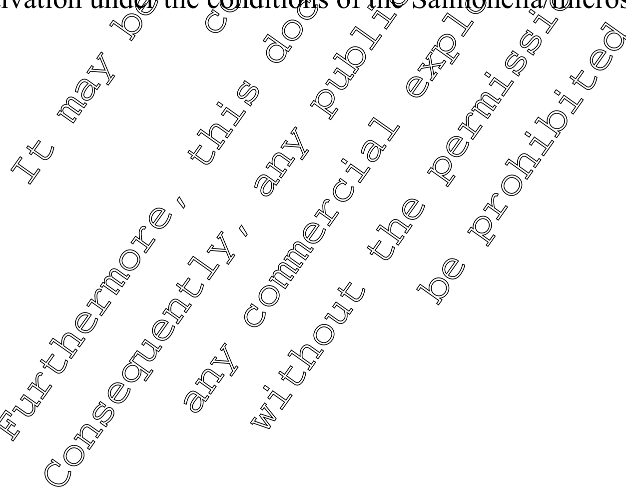
In the 1st trial, the positive controls for both with and w/o activation in the TA102 assay did not fulfill this requirement. Therefore, the assay was performed again using this strain only. The data in the results section reflect the outcome of this assay.

II. Results and Discussions:

Findings:

Doses up to and including 158 µg per plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At higher doses, the substance had a strong, strain-specific bacteriotoxic effect, so that this range could only be used up to and including 1,581 µg per plate for assessment purposes. Evidence of mutagenic activity of BYI 08330 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls had a marked mutagenic effect.

III. Conclusions: BYI 08330 was considered to be non-mutagenic without and with metabolic activation under the conditions of the Salmonella/microsome test.



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Report:	**KIIA 5.4.1/02, 2006**
Title:	BYI 08330 – Salmonella/microsome test plate incorporation and preincubation method
Report No &	AT03070
Document No	M-272000-01-2
Guidelines:	OECD Guideline No. 471, EEC B.13/14, US EPA 712-C-98-247 OPPTS 870.5100. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

A 2nd reverse gene mutation assay was conducted using a batch with an impurity spectrum which was comparable to the proposed technical specification for which approval is applied for. In this reverse gene mutation assay in bacteria, histidine-auxotrophic Salmonella typhimurium LT2 mutant strains TA 100, TA 1535, TA 98, TA 1537 and TA 102 were exposed to BYI 08330, purity 95.2%, batch no. SAV5505-085/2, using dimethylsulfoxide (DMSO) as solvent. The Salmonella/microsome plate incorporation test was initially investigated for point mutagenic effects at doses of 16, 50, 158, 500, 1,581 and 5,000 µg/plate on the five Salmonella strains with and without S9 mix. The independent repeat was performed as preincubation for 20 minutes at 37°C.
Doses up to and including 158 µg per plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At higher doses, the substance had only a weak, strain-specific bacteriotoxic effect. Due to the weakness of this effect this range could nevertheless be used up to 500 µg per plate and partly also up to and including 1,581 µg per plate.
Evidence of mutagenic activity of BYI 08330 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed.
The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant colonies compared to the corresponding negative controls.

Therefore, the BYI 08330 batch for which approval is applied for, was considered also to be non-mutagenic without and with S9 mix in the plate incorporation as well as in the preincubation modification of the Salmonella/microsome test.

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I. Material and methods

A. Materials:

1. Test Material:	BYI 08330
Description:	Technical grade, white powder, room temp storage
Lot/Batch No.:	SAV5505-085/2
Purity (ai%):	95.2% (29-Mar-06)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4,5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	203313-25-1

2. Control Materials:

Negative:	NA	
Solvent (final conc'n):	DMSO, (0.1 ml/plate)	
Positive:	**Nonactivation:**	
	Sodium azide	10 µg/plate TA1535
	Nitrofurantoin	0.2 µg/plate TA 100
	4-Nitro-1, 2- phenylene diamine	0.5 µg/plate TA 98
	4-Nitro-1, 2- phenylene diamine	10 µg/plate TA1537
	Mitomycin C	0.2 µg/plate TA 102
	Cumene hydroperoxide	50 µg/plate TA 102
	Activation: 2-Aminoanthracene	3 µg/plate, all strains

3. Activation: S9 derived from

x induced	x Aroclor 1254	x Rat	x Liver
non-induced	Phenobarbitol	Mouse	Lung
	None	Hamster	Other *[name]*
	Other *[name]*	Other *[name]*	

Describe S9 mix composition

Cofactor solution: $MgCl_2$ x 6 H_2 162.6 mg, 70 mls, S9 fraction, 10 ml, KCl 0.15 M, 20 ml

KCl	246.0 mg
Glucose-6-phospate, disodium salt	179.1 mg
NADP, disodium salt	315.0 mg
Phosphate buffer	100.0 mM

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4. Test organisms: ***S. typhimurium*** **strains** ***[mark those that apply with x]***

	Strain		Strain		Strain		Strain		Strain
	TA97	x	TA98	x	TA100	x	TA102		TA104
x	TA1535	x	TA1537		TA1538		list any others		

Properly maintained?	x Yes	No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	x Yes	No

5. Test compound concentrations used:

Nonactivated conditions:
0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment, 2 trials
Activated conditions:
0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment, 2 trials

B. Tester performance

1. Type of *Salmonella* assay:
_x_ standard plate test
_x_ pre-incubation (20 minutes), followed by plate incorporation, 2nd trial
__ "Prival" modification *(i.e. azo-reduction method)*
__ spot test
__ other

2. Protocol
In the first trial, a plate incorporation method was employed (Ames *et al.* (1973) *Proc. Nat. Acad. Sci.* 70: 2281-2285, Ames *et al.* (1975) *Mutation Res.* 31: 347-364). For the mutant count, three plates were used, both with and without S9 mix, for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control. Each positive control also contained three plates per strain. The amount of solvent for the test substance and for the controls was 0.1 ml/plate. The doses for the first trial were routinely determined on the basis of a standard protocol: if not limited by solubility 5000 µg or 5 µl per plate were used as the highest dose. At least five additional doses were routinely used. If less than three doses were used for assessment, at least used for assessment, at least two repeats were performed. The results of the first experiment were then considered as a pre-test for toxicity. However, in case of a positive response or if at least three doses could be used for assessment, the first trial was included in the assessment. If the second test confirmed the results of the first, no additional repeat was performed. Doses of repeats were chosen on the basis of the results obtained in the first experiment. The independent repeat was performed as preincubation 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. For the mutant count, three plates were used for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control.

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Each positive control also contained three plates per strain. The doses of this trial were determined on the basis of the results of the plate incorporation assay. Doses are given as ug/tube for better separation of plate incorporation and preincubation trials, despite the fact that ug/plate and ug/tube could be used synonymously. The toxicity of the substance was assessed in three ways. The first method was a gross appraisal of background growth on the plates for mutant determination. If a reduction in background growth was observed, it was indicated in the tables by the letter "b" after the mutant count. Where only a single "b", without any other values is noted for a concentration, this "b" represents three plates with reduced background growth. (The same applies to the signs "c", "v", "p", "n" or" %", which may also be used in the tables). Secondly, a toxic effect of the substance was assumed when there was a marked and dose-dependent reduction in the mutant count per plate, compared to the negative controls. Thirdly, the titer was determined. Total bacterial counts were taken on two plates for each concentration studied with S9 mix. However, if an evaluation was performed only without S9 mix, the bacterial count was taken without S9 mix. The bacterial suspensions were obtained from 17-hour cultures in nutrient broth, which had been incubated at 37°C and 90 rpm. These suspensions were used for the determination of mutant counts. No standardized procedure was employed to set the bacterial suspensions at a defined density of viable cells per milliliter, since the chosen method of incubation normally produces the desired density. However, the numbers of viable cells were established in a parallel procedure by determining the titers. The dilution of bacterial suspensions used for the determination of titers was 1:1,000,000. Titers were determined under the same conditions as were the mutations, except that the histidine concentration in the soft agar was increased five-fold to permit the complete growth of bacteria. The tests were performed both with and without S9 mix. The count was made after the plates had been incubated for 48 hours at 37°C. If no immediate count was possible, plates were temporarily stored in a refrigerator.

3. Statistical Analysis: No statistical analysis was performed on the study data.

4. Evaluation Criteria:

The following criteria determined the acceptance of an assay:

a) The negative controls had to be within the expected range, as defined by published data and/ or the laboratories' own historical data; b) the positive controls had to show sufficient effects, as defined by the laboratories' experience; and c) titer determinations had to demonstrate sufficient bacterial density in the suspension. Only trials which complied with all three of the above criteria were accepted for assessment. Even if the criteria for points (b) and (c) were not met, a trial was accepted if it showed mutagenic activity of the test compound. Furthermore, an unacceptable trial would have been repeated.

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II. Results and Discussions:

Findings:

Doses up to and including 158 µg per plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At higher doses, the substance had only a weak, strain-specific bacteriotoxic effect. Due to the weakness of this effect this range could nevertheless be used up to 500 µg per plate and partly also up to and including 1,581 µg per plate. Evidence of mutagenic activity of BYI 08330 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant colonies compared to the corresponding negative controls.

III. Conclusions: BYI 08330 was considered to be non-mutagenic without and with metabolic activation under the conditions of the Salmonella/microsome test.

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IIA 5.4.2 *In vitro* genotoxicity - Test for clastogenicity in mammalian cells

Report:	**KIIA 5.4.2/01, [redacted]; 2002**
Title:	BYI 08330 – In vitro chromosome aberration test with Chinese Hamster V79 cells
Report No & Document No	AT00055 M-065342-01-2
Guidelines:	OECD Guideline No. 473, EEC B.10, US EPA 712-C-98-223 OPPTS 870.5375. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a chromosome aberration test in vitro, Chinese Hamster V79 cells were exposed to BYI 08330, purity 96.5% (analysed December 2001) and 93.5 % (analysed June 2002), batch No. NLL6425-9, using dimethylsulfoxide (DMSO) solvent at concentrations of 10, 30 and 50 µg/ml (4 hours treatment) and 12, 24 and 48 µg/ml (18 hours treatment) in the absence of S9 mix. In the presence of S9 mix, 20, 40 and 80 µg/ml of BYI 08330 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 50 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 80 µg/ml.
Without S9 mix cytotoxic effects were observed at 50 ug/ml and above after 4 hours treatment and at 24 ug/ml and above after 18 hours treatment. With S9 mix cytotoxic effects were observed at 40 ug/ml and above. Precipitation in the medium was not observed. However, metaphase quality interfered with scoring in the respective highest treatment concentration of all trials.

Statistically significant but only weakly increased numbers of aberrant metaphases were noted in cultures harvested 18h after the beginning of a 4h treatment at the highest concentration in the absence (50 µg/ml) and in the presence of S9 mix (80 µg/ml).
Statistically significant but only weakly increased numbers of aberrant metaphases were noted in cultures harvested 30h after the beginning of a 4h treatment at the highest concentration in the presence of S9 mix (80µg/ml), but not in the absence of S9 mix (50 µg/ml).
Statistically significant but only weakly increased numbers of aberrant metaphases were noted in cultures harvested 18h after the beginning of a 18h treatment at the highest concentration in the absence of S9 mix (48µg/ml).

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.

Based on this test, BYI 08330 is considered to be weakly clastogenic for mammalian cells in vitro.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	technical grade, white powder
Lot/Batch No.:	NLL 6425-9
Purity (%ai):	96.5% (11-Dec-01); 93.5% (21-Jun-02)
Compound Stability	room temperature storage
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	203313-25-1
Solvent Used:	DMSO

2. Control Materials:

Negative:	Culture Medium
Solvent (final conc'n):	DMSO (0.2 ml solvent / culture)
Positive:	Nonactivation: Mitomycin C (0.1 µg/ml medium) Activation: Cyclophosphamide (2 µg/ml medium)

3. Activation: S9 derived from

x	induced	x	Aroclor 1254	x	Rat	x	Liver
	non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other		Other		

Describe S9 mix composition:

For use, frozen aliquots of the S9 fraction were slowly thawed and mixed with a cofactor solution (4:6). The S9 mix contained 40% S9 fraction and was kept on ice and used on the same day.

Cofactor solution per 100 ml S9 mix:

Sodium phosphate buffer (100 mM; pH 7.4)	60.0 ml
$MgCl_2$ x 6 H_2O	162.6 mg
KCl	246.0 mg
Glucose-6-phosphate (disodium salt)	152.0 mg
NADP (disodium salt)	315.2 mg

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4. Test cells: mammalian cells in culture

V79 cells were obtained from [redacted], Germany			
Media: Eagle's minimal essential media (MEM)			
Properly maintained?	x	Yes	No
Periodically checked for Mycoplasma contamination?	x	Yes	No
Periodically checked for karyotype stability?	x	Yes	No

5. Test compound concentrations used:

Nonactivated conditions:	Cytotoxicity: 0 (solvent control), 1, 5, 10, 50, 100, 250, 500, 750 µg/ml Main study: (4 hrs. treatment time) 0 (negative), 0 (solvent control), 10, 20, 30, 50, 70 µg/ml; (18 hrs. treatment time) 0 (negative), 0 (solvent control), 6, 12, 24, 36, 48 µg/ml
Activated conditions:	Cytotoxicity: 0 (solvent control), 1, 5, 10, 50, 100, 250, 500, 750 µg/ml Main study: (4 hrs. treatment time) 0 (negative), 0 (solvent control), 20, 40, 60, 80, 100 µg/ml.

B. Test performance

1. Preliminary Cytotoxicity Assay:
1x10^6 cells were seeded in 20 ml of medium on the day prior to treatment. Immediately prior to treatment, this medium was removed from the culture and replaced with 20 mls of MEM plus 2% FCS and 0.2 ml of test material solution (non-activation) or 19 mls of MEM plus 2% FCS, 1 ml of S9 mix and 0.2 ml of test material. After 4 hours of incubation at 37°C, the medium was removed and the cells washed once with pre-warmed phosphate buffered solution. After the addition of MEM plus 10% FCS, the cells were incubated for an additional 18 hours. Two hours prior to the cell harvest, 0.2 ml of Colcemid solution (40 µg/ml) was added to each culture. The cells were thereby arrested in the metaphase of mitosis. Duplicate cultures of each treatment level were performed. The percentage of mitotic indices and the percentage of cell survival were used as criteria to determine cytotoxicity.

2. Cytogenetic Assay:

	a. Cell exposure time:	Test Material	Solvent Control	Positive Control
	Non-activated:	4 h	4 h	4h
	Activated:	4 h	4 h	4h

b. Spindle inhibition

Inhibition used/concentration:	Colcemid, 40 µg/ml
Administration time:	2 hours (before cell harvest)

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c.	**Cell harvest time after termination of treatment:**	Test Material	Solvent Control	Positive Control
	Non-activated:	4 h	4 h	4 h
	Activated:	4 h	4 h	4 h

d. Details of slide preparation:

The medium was removed from each flask and cells were removed from the bottom of the flask by trypsinization and suspended in medium. This medium was transferred to a centrifuge tube and spun for approximately 5 minutes at 700 rpm. The supernatant was carefully removed, 1-2 ml of a hypotonic solution (0.4% KCI; 37°C) was added to the tube. Within 4 minutes, the volume was brought to 6 ml with additional hypotonic solution and cells were resuspended. The cells were sedimented in the centrifuge as before and the supernatant was removed. A few drops of cold (4°C) fixative [ethanol/acetic acid (3:1)] were added and mixed carefully with the cells. The volume was adjusted to 6 ml with the fixative and mixed again with the cells. The mixture was incubated at room temperature for 20 minutes. Cells were pelleted as before and the supernatant was discarded. Cells were again resuspended in fixative as before and centrifuged. Pelleted cells were resuspended carefully in a small volume of fresh fixative. This suspension was dropped onto clean slides which had been frozen at -20°C previously. The slides were allowed to dry for at least 2 hours. Thereafter, they were submerged in pure methanol for 3 minutes and stained for 20-30 minutes in 5% 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. All solutions used during this preparation were freshly prepared each time. The Giemsa solution was filtered before usage.

e. Metaphase analysis

No. of cells examined per dose:			
Scored for structural?	x	Yes	No
Scored for numerical?	x	Yes, polyploidy metaphases were recorded	No
Coded prior to analysis?	x	Yes	No

f. Evaluation criteria:

Coded slides were evaluated using a light microscope at a magnification of about 630. The mitotic index was determined by counting 1000 cells per culture. The numbers of mitotic and non-mitotic cells were noted. Duplicate cultures were processed and examined. 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. In most cases at least 100 assessable metaphases were present on one slide prepared from an individual culture. Therefore, the back-up slide which was generated routinely from every culture was normally not utilized for the evaluation. However, in cases when fewer than 100 assessable metaphases were found on the first slide of a culture, the back-up slides were evaluated as well until a total of 100 metaphases was reached.

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Only metaphases containing the modal chromosome number (22) were analyzed unless exchanges were detected. In this case, metaphases were evaluated even if the chromosome number was not equal to 22. The classes of structural chromosome damage were defined and recorded by using essentially the terminology of [redacted] (1967). Both chromatid and chromosome-type aberrations were assessed. Chromatid-type aberrations are clastogenic effects restricted to one of the two corresponding chromatids. Chromosome type aberrations are defined as changes expressed in both corresponding sister chromatids at the same locus. The distinction between chromatid and chromosome type aberrations was not made for exchanges. The different classes of aberrations were characterized as follows: 1) Gap: A gap is an achromatic lesion within a chromatid arm without obvious dislocation of the chromatid end(s) and smaller than the width of one chromatid. Gaps are found on one chromatid ("gap") or on both chromatids at apparently identical sites ("isogap"). The biological relevance of gaps of both types is unclear. 2) Break: a break is defined as a discontinuity of one chromatid ("break") or both chromatids, at apparently the same locus ("isobreak"), with dislocation of the chromatid ends. The dislocated chromatid end(s) has (have) to be present within the respective metaphase. In addition, an achromatic lesion within a chromatid arm without obvious dislocation of the chromatid end(s) but larger than the width of one chromatid is also defined as break or as isobreak, if this occurs in parallel on both chromatids of a chromosome. 3) Fragment: Fragments are parts of chromosomes without centromer. A fragment is the result of a break. The corresponding defective chromosome is not detectable among the chromosomes of the same metaphase. Fragments can be derived from one chromatid ("fragment") or from both corresponding chromatid regions of a chromosome ("isofragment"). 4) Deletion: A deletion occurs as the result of a break. In case of a deletion, one chromatid ("deletion") or both corresponding terminal chromatid parts of a chromosome ("isodeletion") are missing within the metaphase under assessment. 5) Exchange: This is an exchange of chromatid-parts between different chromosomes (interchange) or within the same chromosome (intrachange). 6) Multiple aberration: A cell was assessed as to contain "multiple aberrations" when five or more structural changes (excluding gaps) occur within one metaphase. In addition to these aberrations, metaphases showing chromosome disintegration as an indication of a cytotoxic effect were also recorded if they were observed. They were counted separately and were not included among the cells that were assessed for aberrations. "Chromosome disintegration" was recorded if fewer than half of the chromosomes reveal characteristic structural features within a given metaphase.

g. Statistical analysis:

The statistical analysis was performed by pair-wise comparison of BYI 08330-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 chi2-test. The numbers of metaphases with aberrations (including and excluding gaps) and of metaphases with exchanges were compared (provided that these data superceded the respective solvent control). Fisher's exact test was used for the statistical evaluation. A difference was considered to be significant, if the probability of error was below 5%.

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II. Results and Discussions:

Findings:
With one exception the cultures treated with BYI 08330 in the absence and in the presence of S9 mix showed for the highest concentration scored statistically significant but only weakly increased numbers of aberrant metaphases. Due to this weakness, these findings need not necessarily to be due to a clastogenic potential but may also be based of an impurity or on a secondary non-genotoxic mechanism.

III. Conclusion: BYI 08330 is considered to be weakly positive in this cytogenetic in vitro study.

Because the previous chromosome aberration test in vitro was done with an earlier less pure material (batch No. NLL6425-9; purity 93.5 – 96.5%), a follow-up *in vitro* screen for clastogenicity was done with later, more highly purified material (NLL 6425-14-a; purity 98.6%; cis/trans 99.2 / 0.8). The negative results of the follow-up study are summarized below (Report No. AT00194).

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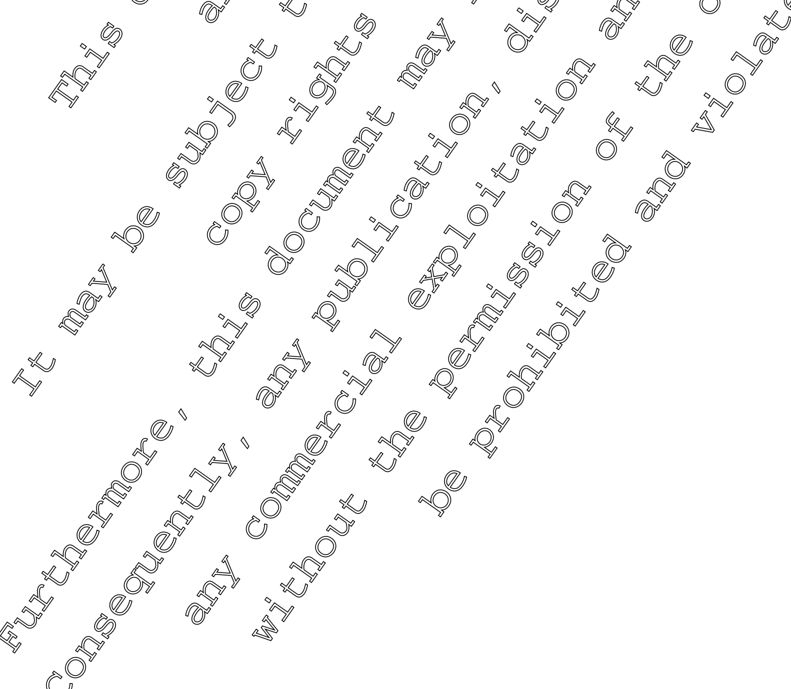
Report:	**KIIA 5.4.2/02, [redacted].; 2006**
Title:	BYI 08330 – Cytogenetic screening with Chinese Hamster V79 cells
Report No &	AT00194
Document No	M-075136-01-2
Guidelines:	OECD Guideline No. 473, EEC B.10, EPA 712-C-98-223 OPPTS 870.5375. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a supplementary chromosome aberration screen in vitro, Chinese Hamster V79 cells were exposed for 4 hours to BYI 08330, purity 98.6%, batch No. NLL 6425-14-a, using dimethylsulfoxide (DMSO) solvent at concentrations of 30, 50, 70, 90 and 110 µg/mL of BYI 08330 without S9 mix and 40, 60, 80, 100 and 120 µg/mL of BYI 08330 with S9 mix. Cultures of all concentrations were harvested 18 hours after the beginning of the treatment. Based on their cytotoxicity concentrations were selected for reading of metaphases.

No biologically relevant or statistically significant increased numbers of aberrant metaphases were noted in cultures harvested 18h after the beginning of a 4h treatment in the absence of S9 mix at 70 µg/ml (GL: 50 µg/ml) and in the presence of S9 mix at 120 µg/ml (GL: 80 µg/ml).

Based on the results of this follow-up screening, BYI 08330 is not considered to have a clastogenic potential on mammalian cells *in vitro,* as these results suggest a cytotoxic impurity was present in the initial batch of BYI 08330 tested. Further support of this conclusion is reflected in the negative *in vivo* findings summarized below (report nos.: AT00048, AR00070, and AT00526).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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

- **Description:** technical, white powder, room temp storage
- **Lot/Batch No.:** NLL 6425-14-a
- **Purity (ai %):** 98.6 (17-Oct-2002)
- **Chemical Name:** 3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4,5]dec-3-en-4-yl carbonate
- **CAS No. of TGAI:** 203313-25-1

2. Control Materials:

- **Negative control:** Culture Medium
- **Solvent control (final conc'n):** DMSO
- **Positive control:** **Nonactivation:** Ethyl methanesulfonate (EMS) (900 µg/ml/ solvent not identified)
  Activation: Dimethylbenzanthracene (DMBA) (20 µg/ml/ solvent not identified)

3. Activation: S9 derived from

x	induced	x	Aroclor 1254	x	Rat	x	Liver
	non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other		Other		

Describe S9 mix composition:
S9 mix was used for the simulation of mammalian metabolism. The S9 fraction was isolated in house from the livers of Aroclor 1254-induced male Sprague Dawley rats. The used S9 fraction was derived from the preparation dated May 13, 2002 (color-code red, protein content 23.1 mg per ml). For use, frozen aliquots of the S9 fraction were slowly thawed and mixed with a cofactor solution (4:6). The S9 mix contained 40% S9 fraction and was kept on ice and used on the same day.

Cofactor solution per 100 ml S9 mix:

Sodium phosphate buffer (100 mM; pH 7.4):	60.0 ml
$MgCl_2 \times 6H_2O$:	162.6 mg
KCl:	246.0 mg
Glucose-6-phosphate (disodium salt):	152.0 mg
NADP (disodium salt):	315.2 mg

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4. Test cells: mammalian cells in culture

V79 cells (Chinese hamster lung fibroblasts): obtained from Germany

Media: Eagle's minimal essential medium (MEM)

Properly maintained?	x	Yes		No
Periodically checked for *Mycoplasma* contamination?	x	Yes		No
Periodically checked for karyotype stability?	x	Yes		No

5. Test compound concentrations used:

Nonactivated conditions:	Cytotoxicity: 0 (solvent control), 1, 5, 10, 50, 100, 250, 500, 750 µg/ml Main study: (4 hrs. treatment time) 0 (negative), 0 (solvent control), 0, 30, 50, 70, 90 µg/ml
Activated conditions:	Cytotoxicity: 0 (solvent control), 1, 5, 10, 50, 100, 250, 500, 750 µg/ml Main study: (4 hrs. treatment time) 0 (negative), 0 (solvent control), 40, 60, 80, 100, 120 µg/ml.

B. Test performance

1. Preliminary Cytotoxicity Assay (from Lab/Study No. T 6071477)

$1x10^6$ cells were seeded in 20 ml of medium on the day prior to treatment. Immediately prior to treatment, this medium was removed from the culture and replaced with 20 mls of MEM plus 2% FCS and 0.2 ml of test material solution (non-activation) or 19 mls of MEM plus 2% FCS, 1 ml of S9 mix and 0.2 ml of test material. After 4 hours of incubation at 37ºC, the medium was removed and the cells washed once with pre-warmed phosphate buffered solution. After the addition of MEM plus 10% FCS, the cells were incubated for an additional 18 hours. Two hours prior to the cell harvest, 0.2 ml of Colcemid solution (40 µg/ml) was added to each culture. The cells were thereby arrested in the metaphase of mitosis. Duplicate cultures of each treatment level were performed. The percentage of mitotic indices and the percentage of cell survival were used as criteria to determine cytotoxicity.

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2. Cytogenetic Assay:

a.	Cell exposure time:	Test Material	Solvent Control	Positive Control
	Non-activated: Activated:	4 h 4 h	4 h 4 h	4 h 4 h

b.	Spindle inhibition	
	Inhibition used/concentration:	Colcemid, 40 µg/ml
	Administration time:	2 hours (before cell harvest)

c.	Cell harvest time after termination of treatment:	Test Material	Solvent Control	Positive Control
	Non-activated: Activated:	14 and 26 h 14 and 26 h	14 and 26 h 14 and 26 h	14 h 14 h

d. Details of slide preparation:

Cells were removed from each flask by trypsinization, suspended in medium, and centrifuged. The resultant pellet was resuspended in a hypotonic solution (0.56% KCl) and recentrifuged. The supernatant was decanted and a cold ethanol/acetic acid (3:1) fixative added and mixed with the cells. After remaining at room temperature for 20 to 30 minutes, the cells were centrifuged again and the supernatant discarded. After an additional treatment with the fixative and a recentrifugation, the pelleted cells were resuspended in a minimal volume of fixative. The suspended cells were then placed on slides which had been pre-cooled to -20° C. The slides were air-dried for at least 2 hours, treated with pure methanol for 3 minutes and stained with a 5% Giemsa solution for 20 to 30 minutes. The slides were then rinsed with water twice, once with acetone, immersed in xylene for 30 minutes and finally dried and covered. Two slides/cultures were made.

e. Metaphase analysis

No. of cells examined per dose: 1000				
Scored for structural?	x	Yes		No
Scored for numerical?	x	Yes, polyploid metaphases were recorded.		No
Coded prior to analysis?	x	Yes		No

f. Evaluation criteria:

The mitotic index was determined by counting the number of mitotic cells out of a total cell count of 1,000 cells per culture. Duplicate cultures for each treatment level were evaluated. One hundred metaphases/cultures (200 metaphases/treatment level) were scored for structural aberrations. The terminology of [redacted] (1967) was used to define the aberrations. Chromatid-type aberrations are clastogenic effects restricted to one of the two corresponding chromatids. Chromosome-type aberrations are defined as changes expressed in both corresponding sister chromatids at the same locus. An increased incidence of gaps noted for either a single chromatid or on both chromatids at identical sites (isogap) without a concomitant increase in other aberrations was not

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considered a clastogenic response. A test was considered to be positive if there was a treatment-related and statistically significant increase in the aberration rate.

g. Statistical analysis:

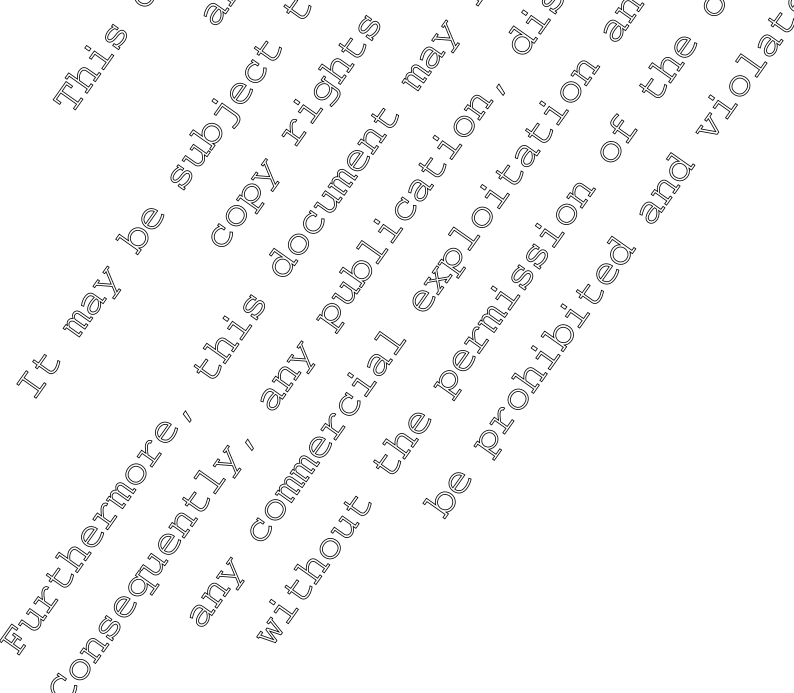
Pair-wise comparisons of test material-treated and positive control groups with the respective control group were performed. The mitotic index values were analyzed for significance by means of the one-sided chi^2-test. The numbers of metaphases with aberrations and of metaphases with exchanges were compared with the respective negative control by using the Fisher's exact test. The probability of error was established at less than 5%.

II. Results and Discussions:

Findings:

Without S9 mix cytotoxic effects were observed at 50 µg/ml and above. With S9 mix cytotoxic effects were observed at 80 µg/ml and above. Precipitation of BYI 08330 in the medium was not observed. In the absence of S9 mix 70 µg/ml BYI 08330 were chosen for reading. In the presence of S9 mix 120 µg/ml of BYI 08330 were employed. None of the cultures treated, both with and without metabolic activation, showed biologically relevant or statistically increased numbers of aberrant metaphases.

III. Conclusion: Based on the results of this follow-up screening, BYI 08330 is not considered to have a clastogenic potential on mammalian cells *in vitro,* as these results suggest a cytotoxic impurity was present in the initial batch of BYI 08330 tested. Further support of this conclusion is reflected in the negative *in vivo* findings summarized just below (report nos.: AT00048, AR00070, and AT00526).



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IIA 5.4.3 *In vitro* genotoxicity - Test for gene mutation in mammalian cells

Report:	**KIIA 5.4.3/01, [redacted].; 2002**
Title:	BYI 08330 – V79/HPRT-Test in vitro for the detection of induced forward mutations
Report No & Document No	AT00137 + Amendment AT00137A of January 2003 M-072857-02-2
Guidelines:	OECD Guideline No. 476, EEC B.17, US EPA 712-C-98-221 OPPTS 870.5300. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

BYI 08330, purity 96.5% (analysed December 2001) and 93.5 % (analysed June 2002), batch no. NLL6425-9, was evaluated for point mutagenic effects at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus (forward mutation assay) in V79 cell cultures in vitro (Chinese hamster lung cells) with concentrations of 2.5, 5, 10, 20, 40 and 80 µg/mL without S9 mix and 20, 40, 60, 80, 100, 120 and 140 µg/mL with S9 mix. The solvent was DMSO.
Without and with S9 mix BYI 08330 induced decreases in survival to treatment and decreases in relative population growth. These results revealed a significant concentration-related cytotoxicity of BYI 08330. Precipitation of BYI 08330 in the culture medium was not observed.

Without and with S9 mix there was no biologically relevant increase in mutant frequency above that of the vehicle 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 08330 is considered to be non-mutagenic in the V79/HPRT Forward Mutation Assay, both with and without metabolic activation.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	technical grade, white powder, room temp storage
Lot/Batch No.:	NLL6425-9
Purity (%ai):	96.5% (11-Dec-01); 93.5% (21-Jun-02)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	203313-25-1
Solvent Used:	DMSO

2. Control Materials:

Negative:	Culture Medium
Solvent (final conc'n):	DMSO (did not exceed 1% (v/v))
Positive:	Nonactivation: Ethyl methanesulfonate (EMS) (900 µg/ml; no solvent required) Activation: Dimethybenzanthracene (DMBA) (20 µg/ml DMSO)

3. Activation: S9 derived from

x	induced	x	Aroclor 1254	x	Rat	x	Liver
	non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other		Other		

Describe S9 mix composition:

Final Concentrations:

$MgCl_2 \times 6H_2O$	8 mM
KCl	33 mM
Glucose-6-phosphate	5 mM
NADP	1 mM
Sodium phosphate	150 mM

40% (v/v) S9 fraction; 60% (v/v) Sodium Phospate buffer

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4. Test cells: mammalian cells in culture

mouse lymphoma L5178Y cells	x	V79 cells (Chinese hamster lung fibroblasts)
Chinese hamster ovary (CHO) cells		list any others

Media: A hypoxanthine-free Eagle's Minimal Essential Medium supplemented with L-glutamine (2mM), MEM-vitamins, $NaHCO_3$, penicillin (100 units/ml), streptomycin (100 µg/ml) and fetal calf serum (10%, heat-inactivated)

Properly maintained?	x	Yes	No
Periodically checked for Mycoplasma contamination?	x	Yes	No
Periodically checked for karyotype stability?	x	Yes	No

5. Locus examinded

		Thymidine kinase (TK)		**Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**	**Na^+/K^+ ATPase**
Selection agent:		bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)	ouabain
		fluorodeoxyuridine (FdU)	x	6-thioguanine (6-TG)	
		trifluorothymidine (TFT)			

6. Test compound concentrations used:

Nonactivated conditions:	0 (negative control), 0 (solvent control), 2.5 thru 80 µg/ml
Activated conditions:	0 (negative control), 0 (solvent control), 20 thru 140 µg/ml

B. Test performance

1. Cell treatment:

Exponentially growing V79 cells were plated in culture medium at a final volume of 20 ml in two 250-ml flasks per concentration ($4x10^6$ per flask) including all control groups. After attachment (16-24 hours later), the cells were exposed for 5 hours in 20 ml culture medium with reduced serum content (2%). The corresponding controls were incubated under the same conditions. Thereafter, cell monolayers were washed with PBS, trypsinized and replated in 20 ml culture medium using 1.5x106 cells per 250 ml flask and in 5 ml culture medium using 200 cells per Petri dish. Per culture one flask and 3 Petri dishes were used. The Petri dishes were incubated (normally 6 days) to allow colony development and to determine the cytotoxicity associated with each test substance directly after treatment ("Survival to Treatment").

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Cells in 250 ml 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 medium in 250 ml flasks. At the end of the expression period (= count 2, normally a total of 6 days), cultures were reseeded in Petri dishes at $3x10^5$ cells per dish (8 dishes per culture) in 20 ml culture medium without hypoxanthine but containing 10 pg/ml 6-TG for selection of mutants. In addition, 200 cells per dish (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. The activation assay was performed independently. The procedure was identical to the nonactivation assay except for the addition of S9 mix. In these experiments 19 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 nonactivation assay.

2. Evaluation Criteria:

Acceptance Criteria:

1) The average cloning efficiency of the negative and vehicle controls should be at least 50%.
2) The average of mutant frequency of the vehicle controls should not exceed $25x10^{-6}$ cells.
3) 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 $5x10^{-6}$.
4) The positive control should induce an average mutant frequency of at least three times that of the vehicle control.
5) 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 negative control.
6) 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.

Assessment Criteria:

1) Mutant frequencies will only be 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.
2) A trial will be considered positive if a concentration-related and in parallel cultures reproducible increase in mutant frequencies is 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 can be reproduced in a second trial, the test substance is considered to be mutagenic.
3) Despite these criteria, a positive result will only be considered relevant, if no significant change in osmolality compared to the vehicle control can be observed. Otherwise, unphysiological culture conditions may be the reason for the positive result.

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4) A test substance will be judged as equivocal if there is no strictly concentration related increase in mutation frequencies but if one or more concentrations induce a reproducible and biologically relevant increase in mutant frequencies in all trials.
5) An assay will be considered negative if no reproducible and relevant increases of mutant frequencies were observed.

3. Statistical Methods:
The statistical analysis relies on the mutant frequencies which are submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights. According to the acceptance criteria, mutant frequencies based on less than 5 plates and/or on a relative survival to treatment and/or a relative population growth and/or an absolute cloning efficiency below 10% are not included in the statistical analysis. The two mutant frequency values obtained per group are, although somewhat related, considered as independent measurements thus increasing the power of the statistical tests applied. Since the protocol of the HPRT assay requires at least two independent trials, the overall analysis without respectively with activation is the most important one for classifying substances into mutagens and non-mutagens. However, separate analyses will be run for each trial in order to examine the consistency of the results. All acceptable groups are included in the weighted analysis of variance followed by pair-wise comparisons to the vehicle control on a nominal significance level of 0.05 using the Dunnett test.

II. Results and Discussions:

Findings:
Without and with S9 mix BYI 08330 induced decreases in survival to treatment and decreases in relative population growth. These results revealed a significant concentration-related cytotoxicity of BYI 08330. Precipitation of BYI 08330 in the culture medium was not observed. BYI 08330 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.

III. Conclusion: BYI 08330 is considered to be non-mutagenic in the V79/HPRT forward mutation assay, both with and without S9 mix.

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IIA 5.4.4 *In vivo* genotoxicity (somatic cells) - Bone marrow or micronucleus

Report:	**KIIA 5.4.4/01, [redacted] 2002**
Title:	BYI 08330 – Micronucleus-Test on the male mouse
Report No & Document No	AT00048 M-065314-01-2
Guidelines:	OECD Guideline No. 474, EEC B.12, US EPA 712-C-98-226,OPPTS 870.5395. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a bone marrow micronucleus assay, BYI 08330, purity 96.5% to 93.5 %, batch no. NLL6425-9, was administered to groups of male mice of the strain Hsd/Win: NMRI by two intraperitoneal injections at target doses of 125, 250 and 500 mg/kg bw, respectively, separated by 24 hours. Cyclophosphamide, the positive substance, was dissolved in deionized water and administered in the same way. Males of the positive control received a single intraperitoneal treatment with 20 mg/kg bw cyclophosphamide. The application volume was 10 ml/kg. The femoral marrow of all groups was prepared 24 hours after the last administration.

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.

Males treated twice with BYI 08330 in doses up to 500 mg/kg showed symptoms of toxicity after administration, starting at 125 mg/kg. These symptoms demonstrate relevant systemic exposure of males to BYI 08330. Two of 10 males died before the end of the test due to the acute intraperitoneal toxicity of 500 mg/kg BYI 08330. In addition, one of five males died in the 250 mg/kg group.

There was an altered ratio between polychromatic and normochromatic erythrocytes. This finding demonstrates relevant systemic exposure of the males to BYI 08330.

After two intraperitoneal treatments of males with doses up to and including 500 mg/kg no biologically relevant indications of a clastogenic effect of BYI 08330 were found.

Based on these results, BYI 08330 is considered not clastogenic in the micronucleus test in male mice.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description: technical grade, white powder, room temp storage

Lot/Batch No.: NLL6425-9

Purity (%ai): 96.5% (11-Dec-01); 93.5% (21-Jun-02)

Chemical Name: 3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate

CAS No. of TGAI: 203313-25-1

2. Control Materials:

Negative control (if not vehicle):	NA	**Final Volume:** NA	**Route:** NA
Vehicle:	0.5% aqueous Cremophor	**Final Volume:** 10 ml/kg	**Route:** i.p.
Positive control:	Cyclophosphamide	**Final Dose(s):** 20 mg/kg	**Route:** i.p.

3. Test animals:

Species: Mouse

Strain: Hsd/Win: NMRI

Age/weight at study initiation: 6 to 12 weeks old, 38 to 44 g.

Source: [redacted] Germany

No. animals used per dose 5 males; 0 females

Properly Maintained? Yes

4. Test compound administration:

	Dose Levels	**Final Volume**	**Route**
Preliminary:	200, 500, 1,000 mg/kg	10 ml/kg	i.p.
Main Study:	0, 125, 250, 500 mg/kg	10 ml/kg	i.p. (2 applications)

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B. Test performance

1. Treatment and Sampling Times:

a. Test compound:

Dosing:		once		twice (24 hrs apart)				Other		
Sampling (after last dose):		24 hr		12 hr		24 hr		48 hr		72 hr
Other:										

b. Negative and/or vehicle control:

Dosing:		once		twice (24 hrs apart)				Other		
Sampling (after last dose):		24 hr		12 hr	x	24 hr		48 hr		72 hr
Other:										

c. Positive control:

Dosing:		once		twice (24 hrs apart)				Other		
Sampling (after last dose):	x	24 hr		12 hr		24 hr		48 hr		72 hr
Other:										

2. Tissues and Cells Examined:

Bone marrow:	Yes
No. of polychromatic erythrocytes (PCE) examined per animal:	2,000
No. of normochromatic erythrocytes (NCE; more mature RBCs) examined per animal:	No. of NCE per 2,000 PCE
Other (if other cell types examined, describe):	NA

3. Details of slide preparation:

At least one intact femur was prepared from each sacrificed animal (not pretreated with a spindle inhibitor). A suitable instrument was used to sever the pelvic bones and lower leg. The femur was separated from muscular tissue. The lower-leg stump, including the knee and all attached soft parts, was separated in the distal epiphyseal cartilage by a gentle pull at the distal end. The proximal end of the femur was opened at its extreme end with a suitable instrument, e.g. fine scissors, making visible a small opening in the bone-marrow channel. A suitable tube was filled with sufficient fetal calf serum. A small amount of serum was drawn from the tube into a suitable syringe with a thin cannula. The cannula was pushed into the open end of the marrow cavity. The femur was then completely immersed in the calf serum and pressed against the wall of the tube, to prevent its slipping off. The contents were then flushed several times and the bone marrow was passed into the serum as a fine suspension. Finally, the flushing might be repeated from the other end, after it had been opened.

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The tube containing the serum and bone marrow was centrifuged in a suitable centrifuge at approximately 1,000 rpm for five minutes. The supernatant was removed with a suitable pipette (e.g. Pasteur pipette), leaving only a small remainder. The sediment was mixed to produce a homogeneous suspension. One drop of the viscous suspension was placed on a well-cleaned slide and spread with a suitable object to allow proper evaluation of the smear. The labeled slides were dried overnight. If fresh smears needed to be stained, they needed to be dried with heat for a short period. The smears were stained automatically with an Ames Hema-Tek Slide Stainer from the Miles Company. The slides were then "destained" with methanol, rinsed with deionized water, and left to dry. Following this treatment, the smears were transferred to a holder. A cuvette was filled with xylene, into which the holder was immersed for approximately ten minutes. The slides were removed singly (e.g. with tweezers) to be covered. A small amount of covering agent was taken from a bottle with a suitable object (e.g. glass rod) and applied to the coated side of the slide. A cover glass was then placed in position without trapping bubbles. The slides were not evaluated until the covering agent had dried.

4. Evaluation Criteria:

Coded slides were evaluated using a light microscope at a magnification of about 1,000. Micronuclei appear as stained chromatin particles in the anucleated erythrocytes. They can be distinguished from artifacts by varying the focus. Normally, 2,000 polychromatic erythrocytes were counted per animal. The incidence of cells with micronuclei was established by scanning the slides in a meandering pattern. It is expedient to establish the ratio of polychromatic to normochromatic erythrocytes for two reasons: 1) individual animals with pathological bone-marrow depressions may be identified and excluded from the evaluation and 2) an alteration of this ratio may show that the test compound actually reaches the target. Therefore, the number of normochromatic erythrocytes per 2,000 polychromatic ones was noted. If the ratio for a single animal amounts to distinctly more than 6,000 normochromatic erythrocytes per 2,000 polychromatic ones, or if such a ratio seems likely without other animals in the group showing similar effects, then the case may be regarded as pathological and unrelated to treatment, and the animal may be omitted from the evaluation. A relevant, treatment-related alteration of the ratio of polychromatic to normochromatic erythrocytes can only be concluded if it is clearly lower for a majority of the animals in the treated group than in the negative control. In addition to the number of normochromatic erythrocytes per 2,000 polychromatic ones, the number of normochromatic erythrocytes showing micronuclei was also established. This information is useful in two ways. Firstly, it permits the detection of individuals already subject to damage before the start of the test. Secondly, combined with the number of micronucleated polychromatic erythrocytes, it permits a representation of the time-effect curve for positive substances. An increase in the number of micronucleated normochromatic erythrocytes, without a preceding increase in micronucleated polychromatic erythrocytes, is irrelevant to the assessment of a clastogenic effect, since normochromatic erythrocytes originate from polychromatic ones. Before an effect can be observed in normochromatic erythrocytes, there must be a much greater increase in micronucleated polychromatic erythrocytes, due to the "dilution effect" of the "old" cells, i.e. normochromatic erythrocytes already present at the start of the test, and this effect would have been observed previously.

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5. Statistical methods:

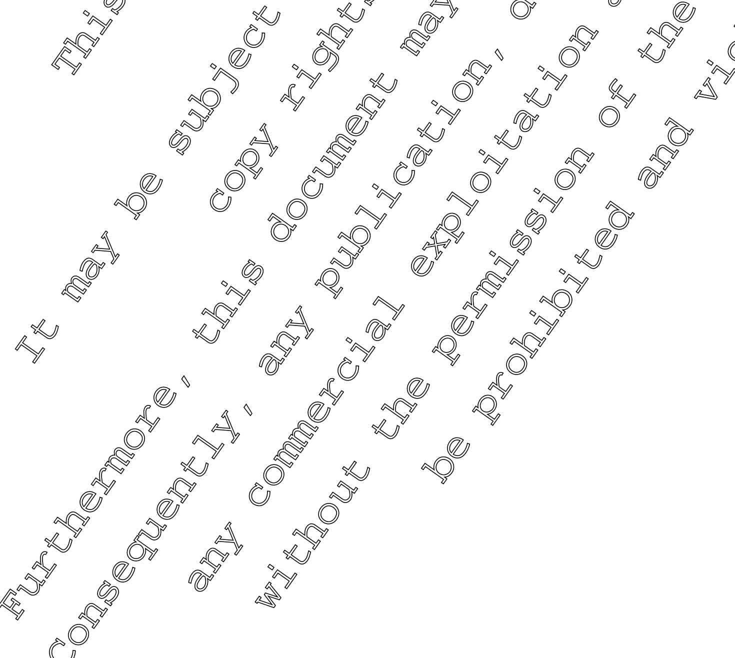
The BYI 08330 group(s) with the highest mean (provided this superceded the negative control mean) and the positive control were checked by Wilcoxon's nonparametric rank sum test with respect to the number of polychromatic erythrocytes having micronuclei and the number of normochromatic erythrocytes. A variation was considered statistically significant if its error probability was below 5% and the treatmentgroup figure was higher than that of the negative control. 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. A variation was considered statistically significant if theerror probability was below 5% and the treatment group figure was higher than that of the negative control. In addition, standard deviations (1s ranges) were calculated for all the means.

II. Results and Discussions:

Findings:

Males treated twice with BYI 08330 in doses up to 500 mg/kg bw showed symptoms of toxicity after administration, starting at 125 mg/kg bw. These symptoms demonstrate relevant systemic exposure of males to BYI 08330. Two of 10 males died before the end of the test due to the acute intraperitoneal toxicity of 500 mg/kg bw BYI 08330. In addition, one of five males died in the 250 mg/kg bw group. There was an altered ratio between polychromatic and normochromatic erythrocytes. This finding demonstrates relevant systemic exposure of the males to BYI 08330. Cyclophosphamide, the positive control, showed a clear clastogenic effect.

III. Conclusion: BYI 08330 was not clastogenic in the micronucleus test in male mice.



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Report:	**KIIA 5.4.4/02, [redacted]; 2003**
Title:	Chromosome aberration assay in bone marrow cells of the mouse with BYI 08330
Report No & Document No	AR00070 M-084116-01-2
Guidelines:	OECD Guideline No. 475, EEC B.11, US EPA 712-C-98-225 OPPTS 870.5385. Deviations: none
OECD/FIFRA GLPS	Yes. Deviations: none

Executive Summary

In a bone marrow cytogenetic assay, BYI 08330, purity 93.5 % (analysed June 2002) and 92.7 % (analysed December 2002), batch no. NLL6425-9, was investigated for the potential to induce chromosome aberrations in bone marrow cells of NMRI male mice. The test substance BYI 08330 was suspended in 0.5% aqueous Cremophor and administered intraperitoneally to the animals at single doses of 125, 250 and 500 mg/kg bw, based on pre-experiments. Cyclophosphamide, the positive control, was dissolved in deoinised water and administered in the same way. Males of the positive control received a single intraperitoneal treatment with 40 mg/kg bw cyclophosphamide. The application volume was 10 ml/kg bw. 24 h and 48 h (only the high dose) after the treatment the bone marrow cells were collected for chromosome aberration analysis.

The positive control, cyclophosphamide, induced a biologically relevant and statistically significant increase of aberrations and thus demonstrated the sensitivity of the test system for the detection of clastogenic effects.

No statistically significant or biologically relevant enhancement of the aberration frequencies occurred after treatment with BYI 08330 as compared to the vehicle control.

In conclusion, BYI 08330 did not induce chromosome mutations and therefore, is considered to be non-mutagenic in this chromosome aberration assay *in vivo*.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

- **Description:** technical grade, white powder, room temperature storage
- **Lot/Batch No.:** NLL 6425-14-a
- **Purity (ai %):** 93.5% (June 21, 2002), 92.7% (December 19, 2002)
- **Chemical Name:** 3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
- **CAS No. of TGAI:** 203313-25-1

2. Control Materials:

Negative control (if not vehicle):		**Final Volume:**	**Route:**
Vehicle:	0.5% aqueous Cremophor	**Final Volume:** 10 ml/kg bw	**Route:** ip
Positive control:	Cyclophosphamide	**Final Dose(s):** 40 mg/kg	**Route:** ip

3. Test animals:

- **Species:** Mouse
- **Strain:** NMRI
- **Age/weight at study initiation:** 8 - 10 weeks old / 38.2 ± 3.2 g
- **Source:**
- **No. animals used per dose** 6 males; 0 females
- **Properly Maintained?** Yes

4. Test compound administration:

	Dose Levels	Final Volume	Route
Preliminary:	500, 1,000 mg/kg bw	10 ml/kg bw	ip
Main Study:	0, 125, 250, 500 mg/kg bw	10 ml/kg bw	ip

B. Test performance

1. Treatment and Sampling Times:

a. Test compound:

Dosing:	x	once		twice (24 hrs apart)				Other (high dose only)		
Sampling (after last dose):	x	24 hr		12 hr		24 hr	x	48 hr		72 hr
Other:										

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b. Negative and/or vehicle control:

Dosing:	x	once		twice (24 hrs apart)				Other		
Sampling (after last dose):	x	24 hr		12 hr		24 hr		48 hr		72 hr
Other:										

c. Positive control:

Dosing:	x	once		twice (24 hrs apart)				Other		
Sampling (after last dose):	x	24 hr		12 hr		24 hr		48 hr		72 hr
Other:										

2. Preparation of the Bone Marrow

The animals were sacrificed by cervical dislocation. The femora were removed, the epiphyses were cut off and the marrow was flushed out with approximately 5 ml hypotonic potassium chloride solution (0.56 % w/v, prewarmed to 37 °C). The hypotonic cell suspension was then incubated for 20 min at 37 °C. The cells were sedimented by a brief centrifugation (1,000 rpm), the hypotonic supernatant was discarded and the cell pellet was fixed with 3+1 absolute methanol+glacial acetic acid fixative for 60 min. Then the cell pellet was gently resuspended with fixative and stored overnight at 4°C. Prior to making slides the fixative was changed and enough fixative was added to make a relatively thin cell suspension. The fixative-cell suspension was spread by flame-drying and stained with Giemsa. Cover slips were mounted with EUKITT (KINDLER, D-79110 Freiburg). One or more slides were made from each bone marrow sample.

3. Analysis of Metaphase Cells

Evaluation of the slides was performed using NIKON microscopes with 100x oil immersion objectives. Gaps, breaks, fragments, deletions, exchanges and chromosomal disintegrations were recorded as structural chromosome aberrations. At least 100 well spread metaphases per animal were scored for cytogenetic damage on coded slides. The number of chromosome aberrations per metaphase was determined. Only metaphases with the characteristic chromosome number of 40$\pm$2 were included in the analysis. To describe a cytotoxic effect, the mitotic index (% cells in mitosis; 1,000 cells are scored) was determined.

4. Evaluation / Acceptance Criteria

The aberration types were defined as follows:

1) Gap: A gap is an achromatic lesion within a chromatid arm without obvious dislocation of the chromatid end(s) and smaller than the width of one chromatid. Gaps are found on one chromatid ("gap") or on both chromatids at apparently identical sites ("isogap"). The biological relevance of gaps of both types is unclear; 2) Break: A break is defined as a discontinuity of one chromatid ("break") or both chromatids, at apparently the same locus ("isobreak"), with dislocation of the chromatid ends. The dislocated chromatid end(s) has (have) to be present within the respective metaphase.

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In addition, an achromatic lesion within a chromatid arm without obvious dislocation of the chromatid end(s) but larger than the width of one chromatid is also defined as break or as isobreak, if this occurs in parallel on both chromatids of a chromosome.
3) Fragment: Fragments are parts of chromosomes without centromer. A fragment is the result of a break.

The corresponding defective chromosome is not detectable among the chromosomes of the same metaphase. Fragments can be derived from one chromatid ("fragment") or from both corresponding chromatid regions of a chromosome ("isofragment"). 4) Deletion: A deletion occurs as the result of a break. In case of a deletion, one chromatid ("deletion") or both corresponding terminal chromatid parts of a chromosome ("isodeletion") are missing within the metaphase under assessment. 5) Exchange: This is an exchange of chromatid-parts between different chromosomes (interchange) or within the same chromosome (intrachange). 6) Multiple aberration: A cell was assessed as to contain "multiple aberrations" when five or more structural changes (excluding gaps) occur within one metaphase. 7) Chromosome disintegration: A chromosome disintegration is recorded if fewer than half of the chromosomes reveal characteristic structural features within a given metaphase. Five animals per group were evaluated as described. The test is acceptable if the positive control shows a statistically significant response and if the aberration rate of the vehicle control (excl. gaps) is below 2 %.

5. Evaluation of Results / Statistical Methods
A test item is classified as mutagenic if it induces either a dose-related increase or a clear increase in the number of structural chromosomal aberrations in a single dose group. Statistical methods (nonparametric Mann-Whitney test) was 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 structural chromosomal aberrations is considered non-mutagenic in this system.

II. Results and Discussions:

Findings:
No substantial reduction of the mitotic indices could be observed after treatment with the test item, indicating that the test item at the doses administered was not cytotoxic in the bone marrow. No biologically relevant or statistically significant increase in the frequency of aberrant cells occurred after treatment with the test item as compared to the vehicle control. The appropriate reference mutagen (cyclophosphamide) showed a distinct and statistically significant increase of induced aberration frequency.

III. Conclusion: BYI 08330 was negative in this chromosome aberration assay in vivo.

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IIA 5.4.5 *In vivo* genotoxicity (somatic cells) - DNA repair or mouse spot tests

Report:	**KIIA 5.4.5/01, [redacted]; 2003**
Title:	BYI 08330 – Unscheduled DNA synthesis test with rat liver cells in vivo
Report No &	AT00526
Document No	M-116087-01-2
Guidelines:	OECD Guideline No. 486. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In an unscheduled DNA synthesis (UDS) test with rat liver cells in vivo, BYI 08330, purity 92.7 % - 93.5 %, batch no. NLL6425-9, was tested in groups of 4 male Wistar rats receiving a single oral dose of 1,000 mg/kg bw or 2,000 mg/kg bw, suspended in 0.5 % aqueous Cremophor emulsion. The application volume was 20 ml/kg bw. The known UDS-inducers 2-Acetylaminofluorene and N,N'-Dimethylhydrazine were administered at a single dose of 100 mg/kg bw and 40 mg/kg bw, respectively. Liver cells of the treatment group animals and the negative and positive controls were prepared 4 hours or 16 hours after compound administration. Liver cells of the positive controls were prepared after 4 hours for N,N'-Dimethylhydrazine and after 16 hours for 2-Acetylaminofluorene.

Positive control treatments induced the appropriate response.

BYI 08330 was evaluated as negative in the UDS assay with hepatocytes of male Wistar rats after single oral exposure via gavage at 1,000 and 2,000 mg/kg bw at both sacrifice time points.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	technical grade, white powder, room temperature storage
Lot/Batch No.:	NLL6425-9
Purity (%ai):	93.5% (6/2002); 92.7% (12/2002)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	203313-25-1
Solvent Used:	DMSO

2. Control Materials:

Negative:	Culture Medium
Positive:	2-Acetylaminofluorene (2-AAF); sacrifice time of 16 hours N,N'-Dimethylhydrazine (DMH); sacrifice time of 4 hours

3. Test animals:

Species:	Rat
Strain:	Wistar rats Crl:(WI)BR
Age/weight at study initiation:	5-7 wks of age and 101-150 g at arrival
Source:	[redacted]
No. animals used per dose	4 males; 0 females
Properly Maintained?	Yes

4. Test compound administration:

Dose Levels	Final Volume	Route
0, 1,000, 2,000 mg/kg	20 ml/kg	p.o.

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B. Test performance

1. Treatment and Sampling Times:

a. Test compound:

Dosing:		once	x	once				Other		
Sampling (after last dose)		24 hr	x	4 hr	x	16 hr		48 hr		72 hr
Other:										

b. Negative and/or vehicle control:

Dosing:		once	x	once				Other		
Sampling (after last dose)		24 hr	x	4 hr	x	16 hr		48 hr		72 hr
Other:										

c. Positive control:

Dosing:	x	once		twice (24 hrs apart)				Other		
Sampling (DMH):	x	4 hr								
Sampling (2-AAF):	x	16 hr								
Other:										

2. Preparation of Hepatocyte Cultures

For each animal, cells were seeded in two 60 mm-Petri dishes (7.5 x10^5 viable cells per dish) precoated with collagen to determine cell viability, attachment rate and morphology about 1.5 hours after establishment of the cultures. For the determination of the genotoxicity of the test substance in the UDS assay a Thermanox 25-mm round plastic covership precoated with collagen was placed into each well of 6-well culture dishes. Approximately 3.75 x 10^5 viable cells were seeded per well (in 2.5 ml culture medium 1), whereby 4 wells per animal including the control groups were established. All cultures were incubated for 90 min in a 37°C incubator in a humidified atmosphere containing approximately 5% CO_2.

3. Culture Labelling

After the attachment period, cell number and viability of the cultures were determined by the method of trypan blue exclusion employing the two additional 60 mm-Petri dishes. Also, following the attachment period, cells in the 6-well dishes were washed with culture medium 2 (culture medium 1 without dexamethasone, containing 1% FCS) and finally incubated in the same medium. To each culture 10 µCi/ml ^{3}H-thymidine (specific activity: 15.8 Ci/mmole and 6.2 Ci/mmole, respectively) was added. The cultures were then placed in the incubator.

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After 4 h incubation hepatocytes were washed twice with culture medium 3 (culture medium 2 without gentamycin sulfate) containing unlabelled thymidine and subsequently cultivated in this medium overnight (cold chase).

Thereafter, the cultures were washed twice with PBS in the 6-well dishes. Subsequently, a 1% sodium citrate solution was added to swell the nuclei for 5-10 min. The cells on the coverslips were then fixed by two changes of a 1:4 acetic acid:absolute ethanol solution for a total fixing time of at least 30 min. Wells were then washed 2-6 times each with deionized distilled water and air dried.

4. Autoradiography and Grain Counting

The entire autoradiography procedure is performed in the dark. Air-dried coverslips were mounted cell-side-up on microscope slides. In a darkroom, these were dipped in a NTB-2 photographic emulsion (Kodak), diluted 1:1 with distilled water, and left to dry in the air overnight. The next day, the coated slides were stored in light-tight boxes in the presence of a drying agent (Drierite) for 14 days at -20°C in order to reduce the CG background and therefore to increase the sensitivity of the assay. The photographic emulsion was then developed for 3-4 min in Kodak D-19 developer at temperatures below 15°C. The slides were rinsed afterwards with distilled water, fixed in Kodak Fixer for 7-8 min, and air dried. Slides were then stained with hematoxylin and eosin. Grain counting was done by eye using a Zeiss microscope interfaced to a TV color screen (Sony) with a Sony high resolution TV color camera. UDS was determined as follows: Evaluation was performed with coded slides. Each slide was examined by counting 50 cells per slide. 3 slides per animal were evaluated (total of 150 nuclei for each rat). Only cells viable at the time of fixation were scored; isolated nuclei and cells with abnormal morphology were excluded. A starting point was randomly selected on the lower third of the slide, and cells were scored in a regular fashion by bringing new cells into the field of view, moving along the X-axis. If the total number of 50 cells had not been reached before coming to the edge of the slide, the stage was moved on the Y-axis, and counting resumed in the opposite X-direction, parallel to the first line. UDS was measured by counting nuclear grains and subtracting the average number of grains in 3 cytoplasmic areas of the same size as the corresponding nucleus. This value was referred to as the nuclear net grain (NNG) count of the cell. The mean NNG count per animal was routinely determined from triplicate coverslips. The mean NNG value per dose group was calculated from the mean NNG value of each animal of the respective dose group. The number of cells in repair (nuclei with 5 or more net grains) was also determined. Grains were counted by eye. Data were transferred to a PC and processed with the released and Windows NT 4.0 based software "UDS-Test" (V02.02) of Bayer AG.

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5. Assay Evaluation Criteria

1. For each of the evaluated 50 cells per slide, the number of nuclear grains is scored, as well as numbers of three cytoplasmic grain counts from nuclear-sized areas adjacent to each nucleus.
2. Only cells viable at the time of fixation and with nuclei evenly coated with emulsion will be scored. Cells with abnormal morphology, such as those with pyknotic or lysed nuclei, will not be counted. Isolated nuclei not surrounded by cytoplasm will not be counted.
3. For the conditions described here, a number of 5 NNG or more is chosen as a conservative estimate as to whether a particular cell is responding (cell "in repair").
4. Cells with heavily labelled nuclei (= S-phase cells) will be excluded from scoring.

6. Statistical methods

Descriptive statistical methods were used to calculate means and standard deviations. The means and standard deviations in the tables were calculated from the means calculated individually for each of the three coverslips per animal. Statistical evaluation of % cells in repair was performed using the Chi^2 test.

Net Grains per Nucleus = average number of the mean nuclear net grain counts of each evaluable coverslip; 50 cells per coverslip

Mean Grains per Nucleus = average number of the mean nuclear grain counts of each evaluable coverslip; 50 cells per coverslip

Mean Cytoplasmic Grain Count = average number of the mean cytoplasmic grain counts (3 areas per cell) of each evaluable coverslip; 50 cells per coverslip.

% Nuclei with 5 or more grains = (number of cells with 5 or more net nuclear grain counts per dose / No. of evaluated cells per dose) x 100

Absolute Survival (%) = % of viable cells after isolation

II. Results and Discussions:

Findings:

Rats treated with BYI 08330 showed symptoms of toxicity after administration of both doses. Signs included roughened fur, pallor (at 2,000 mg/kg only) and rapid breathing. No relevant cytotoxicity was observed in hepatocytes after isolation. After single oral treatment with 1,000 mg/kg bw and 2,000 mg/kg bw BYI 08330, respectively, no indication of UDS-induction was found at both sacrifice time. The positive controls 2-Acetylaminofluorene and N,N'-Dimethylhydrazine had clear genotoxic effects.

III. Conclusion: BYI 08330 was negative in the in vivo UDS assay with rat liver cells.

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IIA 5.4.6 *In vivo* studies in germ cells

Not required

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IIA 5.5 Long-term toxicity and carcinogenicity

The results from the long-term toxicity studies in rats and mice confirm the toxicological profile established in the short-term toxicity studies. Rats and mice tolerated the chemical without increased mortality rates and without distinct clinical signs at doses up to a maximum tolerated dose (rat) or in excess of the limit dose (mouse).
Body weight was significantly decreased in rats of both sexes at the highest dietary exposure level of the 2-year carcinogenicity study. At approximately 1 year (day 371), body weight decreases of 6.1% and 13.2% were noted for 7,500-ppm males and 12,000-ppm females, respectively. At approximately 2 years (day 714), body weight decreases of 10.2 and 14.2% were noted for 7,500-ppm males and 12,000-ppm females, respectively, indicating that a maximum tolerated dose was reached.
In the 1-year chronic study, utilizing the same dose regimen, body weight decreases of 6.6 % were noted in 12,000-ppm females at end of treatment. Male body weights were not affected.

A chronic (12-months) feeding study was conducted in the rat with dietary concentrations of 0, 250, 3,500, or 7,500 ♂/ 12,000 ♀ ppm. Effects in males included increased incidences of alveolar macrophage accumulation at 3,500- and 7,500-ppm and slightly increased relative liver weights at 7,500 ppm. There was no evidence of testicular toxicity in a total number of 25 male rats at 7,500 ppm (equal to 414 mg/kg bw/day) examined histopathologically after one year of treatment. Open field assessments conducted once a week and functional observations conducted at the end of the treatment period revealed no evidence for a neurotoxic potential of BYI 08330.
The chronic (12 months) NOAEL in male rats was established at 250 ppm (equal to 13.2 mg/kg bw/day).
In 12,000-ppm females effects included decreased terminal body weights, increased incidences of stains in the perigenital/tail area and discoloration of the lung , increased relative liver weights, and increased incidences of alveolar macrophage accumulation. The chronic (12 months) NOAEL in female rats was established at 3,500 ppm (equal to 255 mg/kg bw/day).

A chronic (24-months) feeding study was conducted in the rat with dietary concentrations of 0, 250, 3,500, or 7,500 ♂/ 12,000 ♀ ppm. After 2 years of treatment, effects at the highest exposure level in both sexes included decreased terminal body weights, increased incidences of stains in the perigenital/tail area, increased incidence of bile duct hyperplasia (females only), decreased absolute kidney weights and increased incidence of renal tubular dilatation in the outer medulla (presumed to be in the thin descending limb of the loop of Henle), increased relative lung weights, discoloured zones in the lung (females only), alveolar macrophage accumulation and a complex of changes described as interstitial pneumonia. Both findings were interpreted as a continuum of morphologic changes. Interstitial pneumonia was diagnosed with the presence of one or more of the following lesions: presence of lymphocytes, cholesterol clefts, interstitial thickening of the alveolar septae by connective tissue, or increase of alveolar pneumocytes and presence of occasional extravasation of erythrocytes (micro hemorrhage). The lung lesions were focal to multifocal in distribution and involved a very small overall portion of the lung tissue; the majority of the lung tissue was characterized as normal.

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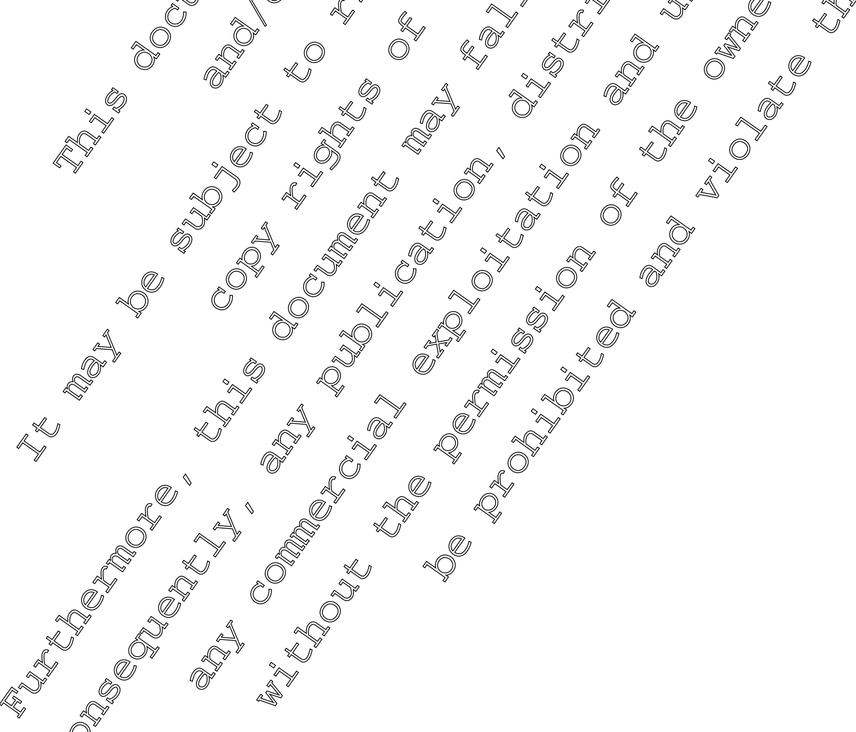
In the testes, an increased incidence of a generally slight morphologic testicular change (more subtle than distinct tubular degeneration) was noted at 7,500 ppm (equal to 373 mg/kg bw/day), characterized by a depletion, asynchrony, and degeneration of the latter stage spermatids. In the epididymis, an increased incidence of immature/exfoliated germ cells/debris was observed, which correlates with the testicular change. There was no morphologic change noted in the anatomical structure of the epididymal tissue.
At 3,500 ppm (equal to 169 / 229 mg/kg bw/day in males / females), decreased absolute kidney weights and an increased incidence of renal tubular dilatation was noted in both sexes.
The chronic (24 months) NOAEL was established at 250 ppm (equal to 12.5 / 16.8 mg/kg bw/day) in male / female rats, respectively.
Based on type, incidence and organ distribution of neoplastic lesions in treated and control rats, there was no indication for an oncogenic effect of BYI 08330.

A chronic (18-months) feeding study was conducted in the mouse with dietary concentrations of 0, 70, 1,700, or 7,000/6,000 ppm. Starting with week 12 of the study, the level of 7,000 ppm was reduced to 6,000 ppm in order to achieve an average active ingredient intake of approximately 1,000 mg/kg bw/day through 18 months of exposure. Unlike the rat no effects of any kind emerged in the mouse, which was tested up to the limit dose in an 18-months feeding study.
The chronic (18 months) NOAEL was established at 6,000 ppm (equal to 1,022 / 1,319 mg/kg bw/day) in males / females, respectively.
Based on type, incidence and organ distribution of neoplastic lesions in treated and control mice, there was no indication for an oncogenic effect of BYI 08330.



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IIA 5.5.1 Long-term (2 years) oral toxicity in the rat

Report:	**KIIA 5.5.1/01, █████.; 2005**
Title:	Technical Grade BYI 08330, A Chronic Toxicity Testing Study in the Rat
Report No & Document No	201285 M-260765-01-1
Guidelines:	OECD 452 (1981), US-EPA OPPTS 870.4100 (1998), JMAFF 12-Nou-san No. 8147; Deviations: none
OECD/FIFRA GLPS	Yes. Deviations: none

Executive Summary

In a chronic (1-year) feeding study, BYI 08330, batch no. 08045/0014, purity: 97.5 – 98.5 %, was administered continuously for up to 54 weeks to 25 male and 25 female Wistar Hanover rats (strain: Crl:WI[Glx/BRL/Han] IGS BR) with their diet at concentrations of 0, 250, 3,500, or 7,500 (males) / 12,000 (females) ppm. The dietary concentrations were equal to doses of 0, 13, 189, and 414 mg/kg bw/day for males and 0, 18, 255, and 890 mg/kg bw/day for females.
Reduced terminal body weight and increased incidences of yellow and brown staining on the perigenital area and tail were noted in females at 12,000 ppm; male body weight was unaffected up to the highest dietary concentration tested. Hematology, clinicochemistry and urinalysis were not affected by treatment. Increased incidence of discoloration of the lung was noted in 12,000-ppm females. Minimal to slight accumulation of alveolar macrophages was observed in the lungs of 7,500-ppm males and 12,000-ppm females and in males of the 3,500 ppm group. Increased relative liver weights were noted in male and female rats of the high dose group (7,500-ppm males) / 12,000-ppm females).
There was no evidence of testicular toxicity in a total number of 25 male rats at 7,500 ppm (equal to 414 mg/kg bw/day) examined histopathologically after one year of treatment. Open field assessments conducted once a week and functional observations conducted at the end of the treatment period revealed no evidence for a neurotoxic potential of BYI 08330.

The chronic (12 months) NOAEL was 250 ppm (equal to 13.2 mg/kg bw/day) in male rats, based on an increased incidence of accumulation of alveolar macrophages at 3,500 ppm (equal to 189 mg/kg bw/day).
The chronic (12 months) NOAEL in females was 3,500 ppm (equal to 255 mg/kg bw/day), based on decreased terminal body weight, yellow and brown staining in the perigenital area and tail, increased relative liver weight, discoloration of the lung and increased incidence of accumulation of alveolar macrophages at 12,000 ppm (equal to 890 mg/kg bw/day).

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	Technical grade, white powder
Lot/Batch No.:	08045/0014
Purity (% a.i.):	97.5% (7/2003); 97.8% (12/2003); 97.6% (6/2004); 98.5% (1/2005)
Compound Stability:	Stable at room temperature (~ 22 ºC)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. for TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1

2. Vehicle and/or positive control: None

3. Test animals:

Species:	Rat; male and female (nulliparous and non-pregnant)	
Strain:	Wistar Hanover rats (Crl:WI[Glx/BRL/Han]IGS BR)	
Age/weight at study initiation:	Approximately 8 weeks old / 241.8 - 243.9g mean group weight males; 154.4 - 157.2g mean group weight females	
Source:	[redacted]	
Housing:	Individually housed in suspended stainless steel wire-mesh cages, each containing a feeder, a source of water (pressure-activated water nipples), and deotized (sanitized) cage board in the bedding tray. One nylabone® was placed in the cage of each animal. Cage racks were rotated monthly.	
Diet:	[redacted] Certified Rodent Diet 5002 in "meal" form; *ad libitum*	
Water:	Municipal water supply of Kansas City, MO; *ad libitum*.	
Environmental conditions:	**Temperature:**	18 to 26 ºC
	Humidity:	30 to 70% (relative); high range relative humidity was 72% on days 24 and 27, and 71% on day 66.
	Air changes:	10.27 changes/hr (minimum daily average)
	Photoperiod:	12 hr of light [7:00 a.m. to 7:00 p.m., or 8:00 a.m. to 8:00 p.m.] alternating with 12 hr of darkness. Deviations in the light/dark cycle occurred 9 times (5 planned; 4 unplanned) over the course of the study. None of the deviations was of a duration as to impact study outcome.
Acclimatisation:	08/25/03 (receipt) - 09/03/03 (release for study)	

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B. Study design:

1. In life dates: Start: 09/03/03 (released for study); 09/09/03 (initiation of exposure)
End: 09/16/04 (terminal sacrifice complete; chronic animals)

2. Animal assignment: 09/04/03; assigned to a control or one of three chemically-treated groups (as noted in Table 1) using a weight stratification-based computer program obtained from INSTEM Computer Systems (Stone, Staffordshire, UK). Each animal on study identified with a microchip ([redacted], DE) subcutaneously implanted on their dorsal surface in the region between the scapulae; the chip was encoded with a unique number, specifying the animal's sex, dose group, cage number, and study affiliation.

Test Group	Conc. in Diet (ppm)	Dose to animal (mg/kg; x±Std)	Main Study 12 months	
			Male	Female
Control	0	0	25	25
Low (LDT)	250	males 13.2 ± 3.4 females 18.0 ± 3.4	25	25
Mid (MDT)	3,500	males 189 ± 49 females 255 ± 39	25	25
High (HDT)	males 7,500 females 12,000	males 414 ± 106 females 890 ± 156	25	25

3. Dose selection rationale:
Doses were based principally upon the toxicological profile which emerged in the rat over the course of a subchronic toxicity testing study (Report No. 201136).
In that study, the toxicological response of the male rat was principally characterized by decreased rate of body weight gain, as well as structural and/or functional alterations in testes- and lung-related endpoints. A decline in rate of body weight gain of 17.6% was noted in 10,000-ppm males; a corresponding decline of 8.4% was noted in absolute body weight. Extrapolating these subchronic changes out over a prolonged chronic exposure (nominally 1 year), which may have increased the severity of the body weight effect, a high-dose of 7,500 ppm was chosen for males in the present study.
In the female, the toxicological response was characterized by a structural and/or functional alteration in the lung (the same lesion noted in 10,000-ppm males). The lung lesion was considered a sign of general toxicity, and progression over the 1-year period could not be excluded. However, unlike the male, a clear body weight effect (a classic predictor of a maximally-tolerated dose; MTD) was not established. These results suggested a different sensitivity in the two sexes, indicating that females could be treated with concentrations somewhat higher than males to achieve roughly the same effect (i.e., males are more sensitive than females). Thus, a high-dose of 12,000 ppm was chosen for females in the present study.

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It was anticipated that the low and high doses chosen of 250 ppm and 7,500 / 12,000 ppm would constitute a *no-observed-adverse-effect level* and a *maximum tolerated dose*, respectively, with the intermediate dosage of 3,500 ppm serving to confirm any dose response relationships that may have emerged.
The probable routes of human exposure to BYI 08330 are via ingestion of foodstuffs that might contain low residues of the test substance, accidental ingestion, or dermal contact during manufacture or use. Thus, formulation with the feed was an appropriate route of administration with which to develop the chronic toxicological profile of the test substance in a given test species.

4. Diet preparation and analysis:
The test compound was mixed directly with the feed; control diet consisted of untreated feed. Samples of each batch of feed mixed were 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 and stored under freezer conditions until presented to the animals the following week.
BYI 08330 was administered at a constant concentration in the feed for the duration of the study. The concentration of the AI of the test substance in the various test diets was analytically verified from samples of each dietary level taken during Weeks 1, 2, 3, 7, 11, 16, 20, 24, 28, 32, 36, 40, 45, and 49. In addition, one recovery sample was analyzed with each group of samples during the test concentration analyses. The homogeneity and stability of BYI 08330 when mixed in the dietary carrier was characterized prior to the in-life phase of the study.
To evaluate the uniformity of distribution/homogeneity of the test substance in the diet, an analysis of feed containing nominal concentrations of 70 and 12,000 ppm BYI 08330 was conducted. Three (3) samples were taken for analysis from each of 3 distinct layers (top, middle, bottom; 9 samples total) in a Hobart mixing bowl. A homogeneous distribution of the test substance in the feed was defined in terms of a percent relative standard deviation (%RSD, same as coefficient of variation; CV), derived from the 9 samples taken, which was ≤ 10%. Additional homogeneity analyses were performed for two other BYI 08330 rodent studies utilizing identical rodent ration mixing procedures in March of 2005. Those studies included separate oncogenicity studies in both rats and mice, and were initiated at about the same time as the present study.
To evaluate the stability of the test substance in the diet, a time and temperature analysis of feed containing nominal concentrations of 70 and 12,000 ppm BYI 08330 was conducted. Stability in the feed was assessed following 1 and 7 days of room temperature storage (~22 ºC) and 7, 14, and 29, and 32 days of freezer storage (~ -23 ºC; 12,000 ppm evaluated at 29 days; 70 ppm evaluated at 32 days). The stability analysis was carried out as follows: An initial sample was taken immediately after the test substance was mixed with the ration. Each batch of ration, mixed at the 2 concentrations, was then divided into 2 portions. One portion was placed in a room temperature environment, the other in the freezer. Feed samples which remained at room temperature were sampled for analysis on Days 0, 1, and 7; feed samples which remained in the freezer were sampled for analysis on Days 7, 14, 29 (12,000 ppm only), and 32 (70 ppm only). Chemical stability in the ration was defined in terms of that period of time that 80% of the test substance, relative to the initial concentration, was recoverable.
The liquid chromatographic methodology used to perform the dietary analyses required for this study (i.e., homogeneity, stability, and concentration verifications) has been described previously.

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Results:

Homogeneity Analysis: The mean concentrations of BYI 08330 in the feed, sampled from 3 distinct layers in the mixing bowl and containing a nominal concentration of either 70 or 12,000 ppm, were determined to be 68.9 ppm (range 64.9-73.2 ppm; %RSD = 3.8%) and 12,157 ppm (range 11,844-12,567 ppm; %RSD = 2.1%), respectively. Based on a %RSD ≤ to 10%, BYI 08330 was judged to be homogeneously distributed in the feed over a concentration range of 70-12,000 ppm. Similarly, additional homogeneity analyses performed for two other rodent studies with BYI 08330 (utilizing identical rodent ration mixing procedures) confirmed the homogeneity of the test substance in the rodent ration.

Stability Analysis: Following 7 days of room temperature storage, the analytically-determined concentration of the AI of the test substance in the 70- or 12,000-ppm admixture was determined to be 69.8 ppm (68.9 ppm on day 0) and 11,948 ppm (12,157 ppm on day 0), respectively. Following 29 and 32 days of freezer storage, the analytically determined concentration of the AI of the test substances in the 12,000- and 70-ppm admixtures was determined to be 12,474 and 68.0 ppm, respectively (12,157 and 68.9 ppm, respectively on day 0). BYI 08330 mixed in rodent ration was judged to be stable at room temperature for at least 7 days and following freezer storage for a minimum 29 days, over a concentration range of 70-12,000 ppm.

Concentration Analysis: Mean analytical concentrations for each dose group were 241, 3,373, 7,366, and 11,549 ppm, ranging from 96-98% of the corresponding nominal concentrations of 250, 3,500, 7,500, and 12,000, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 97% and ranged from 87-104% for rodent ration spiked with 250, 3,500, 7,500, or 12,000 ppm BYI 08330.

5. Statistics:

Continuous data that were examined statistically were evaluated for equality or homogeneity of variance using Bartlett's test. Group means were further analyzed by a one-way variance analysis (ANOVA) followed by Dunnett's test. Frequency data that were examined statistically were evaluated using the Chi-square and/or Fisher exact tests. Continuous data collected in the FOB that were examined statistically were evaluated using an ANOVA with post-hoc comparisons using Dunnett's test. Categorical data collected in the FOB that were examined statistically were evaluated using General Linear Modeling and Categorical Modeling (CATMOD) Procedures, with post-hoc comparisons using Dunnett's test and an Analysis of Contrasts, respectively. For the Bartlett test, a probability (p) level ≤ 0.001 was considered significant; for all other statistical tests, differences with p values ≤ 0.05 were considered statistically significant. Additional statistical tests may have been used to evaluate data generated from this study when deemed appropriate by the study director. All statistical evaluations were performed using software obtained from either INSTEM Computer Systems or SAS Institute Inc..

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C. Methods:

1. Observations:

1a. Cageside Observations:

For 52 weeks of the study, a general assessment of all remaining animals was carried out at least daily (generally twice daily during the normal work week and once daily on weekends and holidays). These assessments were generally with respect to moribundity and mortality, but also included a more general clinical observation for readily apparent clinical signs (in addition to moribundity and mortality).

1b. Clinical Examinations:

Once each week for 52 weeks of the study, detailed physical examinations for clinical signs of toxicity were performed on all surviving animals. These examinations included evaluation of external surface areas, orifices, posture, general behavior, respiration, and excretory products, and a general open field assessment (qualitative; utilizing a standard arena). General observations in the standard arena were not required during the week in which the animals are subject to a more detailed open field observation as part of the full functional observational battery.

1c. Neurological Evaluations:

During month 12 of the study, the last 10 surviving rats/sex/dose were subject to a functional observational battery to assess motor activity, grip strength, and sensory reactivity to stimuli of different types (e.g., visual, auditory, and proprioceptive stimuli). Parameters evaluated included *home cage observations* - posture, piloerection, involuntary motor movements (clonic or tonic), gait abnormalities, vocalizations, decreased activity, repetitive head bobbing (nutation), increased reactivity; *observations during handling* - ease of removing the rat from its cage, reaction to being handled, muscle tone, palpebral closure, lacrimation, salivation, nasal discharge, stains, alopecia, emaciation, bite marks, exophthalmia, broken teeth/malocclusion, missing toe nails, dehydration, cool-to-touch; *open field observations* - piloerection, respiratory abnormalities, posture, involuntary motor movements-clonic or tonic, stereotypy, bizarre behavior, gait abnormalities, vocalizations, rearing, defecation, urination; and *reflex/physiologic observations and measurements* - approach response, touch response, auditory response, tail pinch response, grip strength, body weight, body temperature). Pupil size and pupil response were evaluated during routine ophthalmic examinations.

2. Body weight:

Individual body weight determinations were carried out weekly for the first 13 weeks of the study and every 4 ± 1 weeks thereafter on all surviving animals. Body weights were also measured immediately prior to all necropsies to allow for calculation of organ to body weight ratios.

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3. Food consumption and compound intake:

Individual food consumption determinations were carried out weekly for the first 13 weeks of the study and every 4 ± 1 weeks thereafter on all surviving animals. In addition, using specifically defined criteria, food consumption data were corrected, as conditions dictated, to account for misleading indications of food intake (i.e., excessive spillage, clogged feeders, etc.). Test substance intake (mg/kg body wt/day) was calculated using weekly mean 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

4. Ophthalmologic examination:

Ophthalmologic exams were conducted on all acclimatized animals prior to exposure, and then again on all surviving animals just prior to termination. Ophthalmological lesions were assigned a semi-quantitative ranking of the severity grade/stage from "Normal (within normal limits)" to the "most severe case (Grade 5)," as described previously.

5. Hematology and Clinical Chemistry:

Blood was collected at approximately 3, 6, and 12 months (± 0.5 month/interval) into the study. Blood was drawn via the orbital sinus following an overnight fast from all surviving animals while under light anesthesia (IsoFlo®; Isoflurane). To the extent possible, 15 of the blood samples were used for hematologic (excluding prothrombin time) and clinical determinations; the remaining blood samples (nominally 10) were used for prothrombin time determinations. Differential blood counts were determined on all animals sacrificed due to moribundity.

5a. Hematology:

X	Hematocrit (Hct)*	X	Leukocyte differential count*
X	Hemoglobin (Hgb)*	X	Mean corpuscular Hgb (MCH)*
X	Leukocyte count (WBC)*	X	Mean corpuscular Hgb concentration (MCHC)
X	Erythrocyte count (RBC)*	X	Mean corpuscular volume (MCV)*
X	Platelet count (PLTS)*	X	Reticulocyte count (Retic)
X	Blood clotting measurements*	X	Erythrocyte morphology
	Thromboplastin time	X	Heinz bodies (HZ)
	Clotting time		
X	Prothrombin time (PT)		

* Recommended for chronic studies based on Guideline 870.4100.
The CHECKED (X) parameters were examined.

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5b. Clinical Chemistry:

	ELECTROLYTES		**OTHER**
X	Calcium (Calc)	X	Albumin (Alb)*
X	Chloride (Cl)	X	Creatinine (Creat)*
	Magnesium	X	Blood urea nitrogen (Urea-N)*
X	Phosphate (Phos)	X	Total Cholesterol (Chol)*
X	Potassium (K)*	X	Globulin (Glob)
X	Sodium (Na)*	X	Glucose, fasting (Gluc)*
	ENZYMES (more than 2 hepatic enzymes)*	X	Total bilirubin (T-Bili)
X	Alkaline phosphatase (ALP)*	X	Total protein (T-Prot)*
	Cholinesterase (ChE)	X	Triglycerides (Trig)
X	Creatine phosphokinase (CK)		Serum protein electrophoresis
X	Lactic acid dehydrogenase (LDH)	X	Uric acid (Uric-A)
X	Alanine aminotransferase (ALT)*	X	Thyroxine (T4)
X	Aspartate aminotransferase (AST)*	X	Triiodothyronine (T3)
	Sorbitol dehydrogenase*	X	Thyroid Stimulating Hormone (TSH)
X	Gamma-glutamyltransferase (GGT)*		
	Glutamate dehydrogenase		
	Cytochrome P-450, hepatic (Cyto P-450)		
	N-Demethylase, hepatic (N-Demeth)		
	O-Demethylase, hepatic (O-Demeth)		
	UDP-Glucuronosyltransferase, hepatic (UDP-GT)		

* Recommended for chronic studies based on Guideline 870.4100.
The CHECKED (X) parameters were examined.

6. Urinalysis:

Urine was collected at approximately 3, 6, and 12 months (+ 0.5 month/interval) into the study. Non-fasted urine was collected on an overnight basis (timed) from the first 10 surviving rats/sex/dose the week prior to the week of bleeding.

X	Appearance*	X	Glucose (Glu)*
X	Volume (UVol)*	X	Ketones (Ket)
X	Specific gravity (Sp.Gr.)*	X	Bilirubin (Bil)
X	pH (pH)*	X	Leukocytes (U-Leu)
X	Sediment (microscopic)	X	Blood (Bld)*
X	Protein (Pro)*	X	Nitrite (Nit)
		X	Urobilinogen (UROB)

* Recommended for chronic studies based on Guideline 870.4100.
The CHECKED (X) parameters were examined.

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7. Sacrifice and Pathology:

7a. Gross pathology:

A complete postmortem examination was conducted on all animals (surviving, found dead, sacrificed *in extremis*) which included (1) documenting and saving all gross lesions, (2) weighing designated organs, and (3) collecting representative tissue specimens for histopathologic evaluation. Animals surviving at termination were sacrificed by CO_2 asphyxiation. Tissues were preserved and organ/body weight ratios were calculated for weighed organs.

	DIGESTIVE SYSTEM		**CARDIOVASC./HEMAT.**		**NEUROLOGIC**
	Bile duct (rat)	X	Aorta*	XX	Brain*+
X	Cecum*	X	Bone marrow*	X	Cerebellum*
X	Colon*	XX	Heart*+	X	Cerebrum-Midbrain*
X	Duodenum*	X	Lymph node, cervical*	X	Medulla/Pons*
X	Esophagus*	X	Lymph node, mesenteric*	X	Eyes*
	Gall bladder (not rat)*	XX	Spleen*+	X	Nerve, optic*
X	Ileum*	XX	Thymus+	X	Nerve, sciatic*
X	Jejunum*			X	Pituitary*
XX	Liver*+		**UROGENITAL**	X	Spinal cord, cervical*
X	Pancreas*	X	Cervix	X	Spinal cord, lumbar*
X	Rectum*	X	Clitoral gland^	X	Spinal cord, thoracic*
X	Salivary glands*	XX	Epididymides*+		
X	Stomach, glandular*	XX	Kidneys*+		**OTHER**
X	Stomach, non-glandular*	X	Mammary gland*	X	Bone, femur
X	Tongue	XX	Ovary*+	X	Bone marrow
X	Tooth	X	Preputial gland^	X	Bone, rib/cc jct
		XX	Prostate*	X	Bone, sternum
	RESPIRATORY	X	Seminal vesicle*	X	Gross lesions and masses*
X	Larynx*	XX	Testicle*+	X	Harderian gland
XX	Lung*	X	Urinary bladder*	X	Joint, fem/tib
X	Nasal structure	XX	Uterus*+	X	Muscle, protocol
X	Nasopharynx*	X	Vagina^	X	Physical Identifier (ID chip)
X	Oral structure			X	Skin, protocol*
X	Trachea*		**GLANDULAR**	X	Zymbal's gland^
		XX	Adrenal gland*+		
		X	Exorbital/lacrimal gland^		
		X	Parathyroid*		
		XX	Thyroid*		

* Required for chronic studies based on Guideline 870.4100.

\+ Organ weights required for rodent studies.

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The CHECKED (X) tissues were collected for histological examination. The organs CHECKED (XX) were also weighed. The organs CHECKED (^) were preserved for possible micropathologic evaluation only.

7b. Micropathology:
With the exception of the physical identifier (microchip), the vagina, and the exorbital/lacrimal, clitoral, preputial, and Zymbal glands (of which the latter 5 were taken and preserved for possible micropathologic evaluation), representative sections of the tissues shown in Table 5 were processed for all control (0 ppm) and high-dose (7,500 / 12,000 ppm) animals, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under the light microscope. Tissues in which compound-related effects were detected were examined at lower doses as necessary to establish no-observed-effect levels. Target organs and gross lesions were examined for all animals. Histopathological lesions were assigned a semi-quantitative ranking of the severity grade/stage from "Normal (within normal limits)" to the "most severe case (Grade 5)," as described previously.

II. Results

Findings:
General observations: Clinical observations were limited to an increased incidence of yellow and brown staining on the perigenital area and tail in 12,000-ppm females. No other treatment-related clinical signs and no substance-related mortality were observed. Neurobehavioral parameters (e.g. weekly open field assessments; FOB conducted during Month 12) provided no indication of a neurological effect attributable to exposure to the test substance. Feed intake was not toxicologically relevantly affected. Body weights were slightly reduced (-6.6%) in 12,000 ppm females at end of treatment. Overall mean body weight gain in this group was 86% of controls. Male body weights were not affected. A separate 2-year oncogenicity study with BYI 08330 in the rat, utilizing the same dose regimen, was conducted concurrently with this 1-year chronic rat study. In the 2-year study, significant decreases in mean body weight (compared to controls) were noted in both sexes at the highest doses tested. At approximately 1 year (day 371), decreases of 6.1 and 13.2% were noted for 7,500-ppm males and 12,000-ppm females, respectively. At approximately 2 years (day 714), decreases of 10.2 and 14.2% were noted for 7,500-ppm males and 12,000-ppm females, respectively.

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Table 5.5.1-1: Mean body weights (g) and cumulative body weight gain (g)

Parameter	Dose Group (ppm)			
	0	250	3,500	7,500/12,000
Males				
Initial body weight	244	243	242	243
Final body weight week 51	544	550	541	536
Body weight gain week 1	31	34	31	30
Body weight gain week 13	185	184	182	177
Body weight gain week 27	239	243	238	235
Body weight gain week 51	300	306	299	293
Females				
Initial body weight	157	154	157	157
Final body weight week 51	295	306	287	276
Body weight gain week 1	16	17	15	15
Body weight gain week 13	74	76	69	67
Body weight gain week 27	101	107	91	87 *
Body weight gain week 51	138	152	130	118

* = $p < 0.05$

Hematology: Not affected.

Clinical chemistry: Not affected.

Urinalysis: Not affected.

Ophthalmology: No treatment-related effects.

Gross necropsy, organ weights: Gross necropsy showed an increased incidence of discoloration of the lung in 12,000-ppm females (0, 1, 0, 5). No other indication of a BYI 08330-induced change was noted in either sex at any dose tested.
An increase in relative liver weights was noted in 7,500-ppm males and in 12,000-ppm females which was considered treatment-related. Relative kidney weights were also slightly increased in females at 12,000 ppm which was considered to be due to chance variation.

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Table 5.5.1-2: Mean absolute (g) and relative (%) organ weights

Parameter	Dose Group (ppm)							
	0		250		3,500		7,500/12,000	
Males								
Final body weight	548.7		553.5		544.0		542.6	
Liver weight, absolute	17.792		17.945		18.599		18.913	
Liver weight, relative	3.240		3.249		3.435		3.530	*
Females								
Final body weight	296.0		306.3		289.0		275.2	
Liver weight, absolute	9.790		10.147		9.820		10.476	
Liver weight, relative	3.321		3.328		3.415		3.820	*
Kidney weight, absolute	2.037		2.057		2.030		2.048	
Kidney weight, relative	0.694		0.678		0.706		0.750	*

* = p < 0.05

Histopathology:

Lung: An increased incidence of minimal to slight accumulation of alveolar macrophages was noted in the lungs of 3,500- and 7,500-ppm males, and 12,000-ppm females. This finding correlates with the discoloration of the lung in the 12,000-ppm females (see above). Alveolar macrophage accumulation is considered a common background lesion in the rat; however, the increased incidence noted in this study was attributable to exposure to the test substance.

No other indication of microscopic changes attributable to exposure to the test substance were observed.

Table 5.5.1-3: Average incidence and severity (figures in parenthesis) of microscopic findings

Parameter	Dose Group (ppm)							
	0		250		3,500		7,500/12,000	
Males								
Lungs, macrophages, alveolar	1 (1.0)		2 (1.0)		6 (1.0)	*	11 (1.2)	*
Females								
Lungs, macrophages, alveolar	5 (1.0)		3 (1.0)		2 (1.0)		21 (1.3)	*

* = p < 0.05

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Conclusions: The chronic NOAEL was established at 250 ppm (equal to 13.2 mg/kg bw/day) in male rats, based on an increased incidence of accumulation of alveolar macrophages at 3,500 ppm (equal to 189 mg/kg bw/day).
There was no evidence of testicular toxicity in a total number of 25 male rats at 7,500 ppm (equal to 414 mg/kg bw/day) examined histopathologically after one year of treatment.

The chronic NOAEL in females was established at 3,500 ppm (equal to 255 mg/kg bw/day), based on decreased body weight gain, yellow and brown staining in the perigenital area and tail, increased relative liver weight, discoloration of the lung and increased incidence of accumulation of alveolar macrophages at 12,000 ppm (equal to 890 mg/kg bw/day).

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IIA 5.5.2 Carcinogenicity study in the rat

Report:	**KIIA 5.5.2/01, [redacted]; 2006**
Title:	Technical Grade BYI 08330 (Common Name Spirotetramat): An Oncogenicity Testing Study in the Rat
Report No & Document No	201358 M-273643-01-1
Guidelines:	OPPTS Guideline No. 870.4200 (1998); OECD Guideline No. 451 (1981); JMAFF 12-Nousan-No. 8147; Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory). Deviations: none

Executive Summary

In a chronic (2-year) feeding study, BYI 08330, batch no.: 08045/0014, purity: 97.4 – 98.5 %, was administered continuously for approximately 24 months to 55 male and 55 female Wistar Hanover rats (strain: Crl:WI[Glx/BRL/Han] IGS BR) with their diet at concentrations of 0, 250, 3,500, or 7,500 (males) /12,000 (females) ppm. The dietary concentrations were equal to doses of 0, 12.5, 169, and 373 mg/kg bw/day for males and 0, 16.8, 229, and 823 mg/kg bw/day for females.
Survival was unaffected by treatment. Body weight declines and increased incidences of yellow and brown staining on the perigenital area and tail were noted in both sexes of the high dose group.
Hematology was not affected by treatment. Increased relative lung weights, focal to multifocal interstitial pneumonia involving a small part of the lung, alveolar macrophage accumulation, discoloured zones in the lung (females only), increased incidence of bile duct hyperplasia (females only), decreased absolute kidney weights and an increased incidence of renal tubular dilatation in the outer medulla were noted at the highest dose tested. Few male rats of the highest dose tested showed a more subtle than distinct tubular testicular degeneration, characterized by a depletion, asynchrony, and degeneration of the latter stage spermatids. In the epididymis of high dose males, an increased incidence of immature/exfoliated germ cells/debris was observed which correlated with the testicular change. At the mid dose, decreased absolute kidney weights and renal tubular dilatation was noted in both sexes.
Based on type, incidence and organ distribution of neoplastic lesions in treated and control rats, there was no indication for an oncogenic effect of BYI 08330.
The chronic (24 months) NOAEL was 250 ppm (equal to 12.5 mg/kg bw/day) for male rats, based on decreased absolute kidney weights and an increased incidence of renal tubular dilatation at 3,500 ppm (equal to 169 mg/kg bw/day).
The chronic (24 months) NOAEL in females was 250 ppm (equal to 16.8 mg/kg bw/day), based on decreased absolute kidney weights and an increased incidence of renal tubular dilatation at 3,500 ppm (equal to 229 mg/kg bw/day).

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	Technical grade, white powder
Lot/Batch No.:	08045/0014
Purity:	97.5% (7/2003); 97.8% (12/2003); 97.6% (6/2004); 98.5% (1/2005); 97.4% (5/2005); 98.1% (11/2005)
Compound Stability	Stable at room temperature (~ 22°C)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1

2. Vehicle and/or positive control: None

3. Test animals:

Species:	Rat; males and females (nulliparous and non-pregnant)
Strain:	Wistar Hanover rats (Crl:WI[Glx/BRL/Han]IGS BR)
Age/weight at treatment initiation:	Approximately 7 weeks old / 204.7 - 206.5 g mean group weight males; 151.1 - 152.2 g mean group weight females
Source:	[redacted]
Housing:	Individually housed in suspended stainless steel wire-mesh cages, each containing a feeder, a source of water (pressure-activated water nipples), and deotized (sanitized) cage board in the bedding tray. One nylabone® was placed in the cage of each animal. Cage racks were rotated monthly.
Diet:	[redacted] Certified Rodent Diet 5002 in "meal" form; *ad libitum*
Water:	Municipal water supply of Kansas City, MO; *ad libitum*.
Environmental conditions:	**Temperature:** 18 to 26 °C **Humidity:** 30 to 70% (relative); high range for relative humidity was 72% on Days 232, 295, 436, and 540; 76% on Day 541; 71% on Days 548 and 562; and 75% on Day 569. **Air changes:** 10.08 changes/hr (minimum daily average) **Photoperiod:** 12 hr of light [7:00 a.m. to 7:00 p.m., or 8:00 a.m. to 8:00 p.m.] alternating with 12 hr of darkness. Deviations in the light/dark cycle occurred 8 times (3 planned; 5 unplanned) over the course of the study. The light cycle is unknown for Days 343 (Edstrom version 5 installed), 378 (from 6 p.m. through 12:00 a.m.), 379 (for approx. 1.5 hours; Edstrom system backup and reconfiguration), and 405 (power outage, smart box damaged by lightning strike). None of the deviations was of a duration as to impact study outcome.
Acclimatisation:	07/07/03 (receipt) - 07/14/03 (animals release for study)

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B. Study design:

1. In-life dates: Start: 07/11/03 (study initiation); 07/14/03 (released for study); 07/16/03 (initiation of exposure; males); 07/17/03 (initiation of exposure; females)
End: 07/21/05 (terminal sacrifice complete)

2. Animal Assignment/Dose Levels: 07/14/03 (weighed) and 07/15/03 (randomized); assigned to a control or one of three chemically-treated groups (as noted in Table 1) using a weight stratification-based computer program obtained from INSTEM Computer Systems (Stone, Staffordshire, UK). Each animal on study was identified with a microchip (IPTT-200; [redacted] [redacted], DE) subcutaneously implanted on their dorsal surface in the region between the scapulae; the chip was encoded with a unique number, specifying the animal's sex, dose group, cage number, and study affiliation.

Test Group	Conc. in Diet (ppm)	Dose to animal (mg/kg; x±Std)	Main Study 24 months	
			Male	Female
Control	0	0	55	55
Low (LDT)	250	males 12.5 ± 4.4 females 16.8 ± 3.9	55	55
Mid (MDT)	3,500	males 169 ± 60 females 229 ± 53	55	55
High (HDT)	males 7,500 females 12,000	males 373 ± 134 females 823 ± 157	55	55

3. Dose Selection:
Doses were based principally upon the toxicological profile which emerged in the rat over the course of a subchronic toxicity testing study.
In that study, the toxicological response of the male rat was principally characterized by decreased rate of body weight gain, as well as structural and/or functional alterations in testes- and lung-related endpoints. A decline in rate of body weight gain of 17.6% was noted in 10,000-ppm males; a corresponding decline of 8.4% was noted in absolute body weight. Extrapolating these subchronic changes out over a prolonged lifetime exposure (nominally 2 years), which may have increased the severity of the body weight effect, a high-dose of 7,500 ppm was chosen for males in the present study.
In the female, the toxicological response was characterized by a structural and/or functional alteration in the lung (the same lesion noted in 10,000-ppm males). The lung lesion was considered a sign of general toxicity, and progression over the 2-year period could not be excluded. However, unlike the male, a clear body weight effect (a classic predictor of a maximally-tolerated dose; MTD) was not established.

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These results suggested a different sensitivity in the two sexes, indicating that females could be treated with concentrations somewhat higher than males to achieve roughly the same effect (i.e., males are more sensitive than females). Thus, a high-dose of 12,000 ppm was chosen for females in the present study.

It was anticipated that the low and high doses of 250 ppm and 7,500 / 12,000 ppm would constitute a *no-observed-adverse-effect level* and a *maximum tolerated dose*, respectively, with the intermediate dosage of 3,500 ppm serving to confirm any dose response relationships that may have emerged. The probable routes of human exposure to BYI 08330 are via ingestion of foodstuffs that might contain low residues of the test substance, accidental ingestion, or dermal contact during manufacture or use. Thus, formulation with the feed was an appropriate route of administration with which to develop the chronic toxicological profile of the test substance in a given test species.

4. Diet preparation and analysis:

The test substance was mixed directly with the feed; control diet consisted of untreated feed. Samples of each batch of feed mixed were 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 and stored under freezer conditions until presented to the animals the following week.

BYI 08330 was administered at a constant concentration in the feed for the duration of the study. The concentration of the AI of the test substance in the control, 250-, 3,500-, 7,500-, and 12,000-ppm test diets was analytically verified from samples taken during Weeks 1, 2, 3, 6, 11, 15, 19, 24, 28, 32, 36, 40, 44, 48, 53, 57, 62, 66, 70, 74, 79, 83, 87, 92, 96, 100, and 105. In addition, one recovery sample was analyzed with each group of samples during the test concentration analyses. The homogeneity and stability of BYI 08330 when mixed in the dietary carrier was characterized prior to the in-life phase of the study and again just prior to termination of the in-life phase of the study.

To evaluate the uniformity of distribution/homogeneity of the test substance in the diet, an analysis of feed containing nominal concentrations of 70 and 12,000 ppm BYI 08330 was conducted. Three (3) samples were taken for analysis from each of three distinct layers (top, middle, bottom; 9 samples total) in a Hobart mixing bowl. A homogeneous distribution of the test substance in the feed was defined in terms of a percent relative standard deviation (%RSD, same as coefficient of variation; CV), derived from the 9 samples taken, which was ≤ 10%. Homogeneity was again analyzed, just prior to termination, using one sample from the top, middle, and bottom mixing bowls containing nominal concentrations of 250 and 12,000 ppm BYI 08330.

To evaluate the stability of the test substance in the diet, a time and temperature analysis of feed containing nominal concentrations of 70 and 12,000 ppm BYI 08330 was conducted. Stability in the feed was assessed following 1 and 7 days of room temperature storage (~22 ºC) and 7, 14, and 29, and 32 days of freezer storage (~ -23 ºC; 12,000 ppm evaluated at 29 days; 70 ppm evaluated at 32 days).

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The stability analysis was carried out as follows: An initial sample was taken immediately after the test substance was mixed with the ration. Each batch of ration, mixed at the 2 concentrations, was then divided into 2 portions. One portion was placed in a room temperature environment, the other in the freezer. Feed samples which remained at room temperature, were sampled for analysis on Days 0, 1, and 7; feed samples which remained in the freezer, were sampled for analysis on Days 7, 14, 29 (12,000 ppm only), and 32 (70 ppm only). Chemical stability in the ration was defined in terms of that period of time that 80% of the test substance, relative to the initial concentration, was recoverable. The liquid chromatographic methodology used to perform the dietary analyses required for this study (i.e., homogeneity, stability, and concentration verifications) has been described previously.

Results:

Homogeneity Analysis: The mean concentrations of BYI 08330 in the feed, sampled from three distinct layers in the mixing bowl and containing a nominal concentration of either 70 or 12,000 ppm, were determined to be 68.9 ppm (range 64.9-73.2 ppm; %RSD = 3.8%) and 12,157 ppm (range 11,844-12,567 ppm; %RSD = 2.1%), respectively. Based on a %RSD ≤ to 10%, BYI 08330 was judged to be homogeneously distributed in the feed over a concentration range of 70-12,000 ppm. Homogeneity analysis conducted just prior to study termination again confirmed homogeneity of the test substance in rodent ration. The mean concentrations of BYI 08330 in the feed, sampled from three distinct layers in the mixing bowl and containing a nominal concentration of either 250 or 12,000 ppm, were determined to be 250 ppm (range 248-254 ppm; %RSD = 1.3%) and 11,288 ppm (range 11,245-11,374 ppm; %RSD = 0.7%), respectively.

Stability Analysis: Following 7 days of room temperature storage, the analytically-determined concentration of the AI of the test substance in the 70- or 12,000-ppm admixture was determined to be 69.8 ppm (68.9 ppm on Day 0) and 11,948 ppm (12,157 ppm on Day 0), respectively. Following 29 and 32 days of freezer storage, the analytically-determined concentration of the AI of the test substance in the 12,000- and 70-ppm admixtures was determined to be 12,474 and 68.0 ppm, respectively (12,157 and 68.9 ppm, respectively on Day 0). BYI 08330 mixed in rodent ration was judged to be stable at room temperature for at least 7 days and following freezer storage for a minimum 29 days, over a concentration range of 70-12,000 ppm.

Concentration Analysis: Mean analytical concentrations for each dose group were 249, 3,407, 7,342, and 11,722 ppm, ranging from 97-100% of the corresponding nominal concentrations of 250, 3,500, 7,500, and 12,000 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 98% and ranged from 88-105% for rodent ration spiked with 250, 3,500, 7,500, and 12,000 ppm BYI 08330.

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5. Statistics:

Continuous data that were examined statistically were evaluated initially for equality or homogeneity of variance using Bartlett's test. Group means were further analyzed by a one-way analysis of variance (ANOVA) followed by Dunnett's test for pair-wise comparisons. Frequency data were initially examined for trends; data suggestive of a potential effect were then statistically evaluated using the chi-square, Fisher exact, or chi-square and Fisher exact tests. On a case-by-case basis, and at the discretion of the study director, data were subject to additional statistical procedures other than those mentioned above (e.g., Thakur "TREND" program, Ajit, K. Thakur). For the Bartlett test, a probability (p) value ≤ 0.001 was considered significant; for all other statistical tests, differences with p values ≤ 0.05 were considered statistically significant. All statistical evaluations were performed using software obtained from either INSTEM Computer Systems or SAS Institute Inc..

C. Methods:

1. Observations -

1a. Cageside Observations:

For approximately 105 weeks of the study, a general assessment of all remaining animals was carried out at least daily (generally twice daily during the normal work week and once daily on weekends and holidays). These assessments were generally with respect to moribundity and mortality, but also included a more general clinical observation for readily apparent clinical signs (in addition to moribundity and mortality).

1b. Clinical Examinations:

Once each week for approximately 104 weeks of the study, detailed physical examinations for clinical signs of toxicity were performed on all surviving animals. These examinations included evaluation of external surface areas (visual and palpation), orifices, posture, general behavior, respiration, and excretory products.

2. Body weight:

Individual body weight determinations were carried out weekly for the first 13 weeks of the study and every 4 ± 1 weeks thereafter on all surviving animals. Body weights were also measured immediately prior to all necropsies to allow for calculation of organ/body weight ratios with the following exceptions. Terminal body weights for animals RK3010 (term sac) and RK3151 (sacrificed *in extremis*) were not measured. Final live body weights were used to calculate organ/body weight ratios as appropriate.

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3. Food consumption and compound intake:

Individual food consumption determinations were carried out weekly for the first 13 weeks of the study and every 4 $\pm$ 1 weeks thereafter on all surviving animals. In addition, using specifically defined criteria, food consumption data were corrected, as conditions dictated, to account for misleading indications of food intake (i.e., excessive spillage, clogged feeders, etc.). Test substance intake (mg/kg body wt/day) was calculated using weekly mean 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

4. Ophthalmologic examination:

Ophthalmologic examinations were not conducted.

5. Hematology & Clinical Chemistry:

Blood was collected at approximately 12, 18, and 24 months into the study from all surviving animals for differential determinations. In all cases, blood was sampled via the orbital sinus from non-fasted animals. Differential blood counts were determined on all animals sacrificed due to moribundity with the exception of RK1022 (had been bled for this purpose earlier in the week; scheduled blood collection).

Clinical Chemistry determinations were not performed.

5a. Hematology:

	Hematocrit (Hct)	x	Leukocyte differential count*
	Hemoglobin (Hgb)		Mean corpuscular Hgb (MCH)
	Leukocyte count (WBC)		Mean corpuscular Hgb concentration (MCHC)
	Erythrocyte count (RBC)		Mean corpuscular volume (MCV)
	Platelet count (PLTS)		Reticulocyte count (Retic)
	Blood clotting measurements	x	Erythrocyte morphology
	Thromboplastin time		Heinz bodies (HZ)
	Clotting time		
	Prothrombin time (PT)		

* Minimum required for carcinogenicity studies (Cont. and HDT unless effects are observed) based on Guideline 870.4200 & OECD 451.

The CHECKED (X) parameters were examined.

6. Urinalysis:

Urinalysis determinations were not performed.

7. Sacrifice and Pathology:

7a. Gross pathology:

A complete postmortem examination was conducted on all animals (surviving, found dead, sacrificed *in extremis*) which included (1) documenting and saving all gross lesions, (2) weighing designated organs, and (3) collecting representative tissue specimens for histopathologic evaluation.

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Animals surviving at termination were sacrificed by CO_2 asphyxiation. Tissues were preserved and organ/body weight ratios were calculated for weighed organs.

	DIGESTIVE SYSTEM		**CARDIOVASC./HEMAT.**		**NEUROLOGIC**
	Bile duct* (rat)	X	Aorta*	XX	Brain*+
X	Cecum*	X	Bone marrow*	X	Cerebellum
X	Colon*	XX	Heart*+	X	Cerebrum-Midbrain
X	Duodenum*	X	Lymph node, cervical*	X	Medulla/Pons
X	Esophagus*	X	Lymph node, mesenteric*	X	Eyes*
X	Gall bladder* (not rat)	XX	Spleen*+	X	Nerve, optic*
X	Ileum*	X	Thymus	X	Nerve, sciatic*
X	Jejunum*			X	Pituitary*
XX	Liver*+		**UROGENITAL**	X	Spinal cord, cervical*
X	Pancreas*	X	Cervix	X	Spinal cord, lumbar*
X	Rectum*	X	Clitoral gland^	X	Spinal cord, thoracic*
X	Salivary glands*	X	Epididymides*+		
X	Stomach, glandular*	XX	Kidneys*+		**OTHER**
X	Stomach, non-glandular*	X	Mammary gland*	X	Bone, femur
X	Tongue	XX	Ovary*+	X	Bone, rib/cc jct
X	Tooth	X	Preputial gland^	X	Bone, sternum
		X	Prostate*	X	Gross lesions and masses*
	RESPIRATORY	X	Seminal vesicle*	X	Harderian gland
X	Larynx*	XX	Testicle*+	X	Joint, fem/tib
XX	Lung*++	X	Urinary bladder*	X	Muscle, protocol
X	Nasal structure*	X	Uterus*+	X	Physical Identifier (ID chip) (1)
X	Nasopharynx*	X	Vagina^	X	Skin, protocol*
X	Oral structure			X	Zymbal's gland^
X	Trachea*		**GLANDULAR**		
		XX	Adrenal gland*+		
		X	Exorbital/lacrimal gland^		
		X	Parathyroid*		
		X	Thyroid*		

* Required for carcinogenicity studies based on Guideline 870.4200.
+ Organ weight required in carcinogenicity studies.
++ Organ weight required if inhalation route.
The CHECKED (X) tissues were collected for histological examination. The organs CHECKED (XX) were also weighed. The organs CHECKED (^) were preserved for possible micropathologic evaluation only.
(1) The ID chip for animal RK1001 was cut during the necropsy process. The tail was marked with an indelible marker and retained as the physical identifier.

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7b. Micropathology:

With the exception of the physical identifier (microchip or tail), the vagina, and the exorbital/lacrimal, clitoral, preputial, and Zymbal glands (of which the latter 5 were taken and preserved for possible micropathologic evaluation), representative sections of the tissues shown in Table 5 were processed for all control (0 ppm) and high-dose (7,500 / 12,000 ppm) animals, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under the light microscope. Tissues in which compound-related effects were detected were examined at lower doses as necessary to establish no-observed-effect levels. Target organs and gross lesions were examined for all animals. Histopathological lesions were assigned a semi-quantitative ranking of the severity grade/stage from "Normal (within normal limits)" to the "most severe case (Grade 5)," as described previously. The "grade number" was used to calculate an average grade for a particular observation within a dose group. Neoplastic lesions were generally not assigned a grade.Tissues from approximately 101 animals were processed as described above by . These animals represented all early deaths through day 682 (05/28/05). All other histology was performed by in-house (Bayer CropScience LP - Toxicology). The initial micropathologic evaluation of this study was performed by (Bayer CropScience LP - Toxicology). An internal peer review was later conducted by , and included examination of various selected and randomly-selected slides.

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II. Results

Findings:

General observations: Survival was unaffected by treatment, as the incidence of mortality was comparable between groups. Early deaths/survival numbers in males were 15/40, 21/34, 11/44, 20/35 and 16/39, 16/39, 18/37, 13/42 in females in ascending order of dose levels. Clinical observations revealed an increased incidence of "body, scaly" and yellow and brown staining on the perigenital, perianal area and tail in 7500 ppm males and 12,000-ppm females. Feed intake was not toxicologically relevantly affected. Significant decreases in mean body weight and body weight gain were noted in both sexes at the highest doses tested. Based on final body weight measurements (week 103), body weight declines of 10 and 14% were seen for 7,500-ppm males and 12,000-ppm females, respectively.

Table 5.5.2-1: Incidence of treatment-related clinical observations

Parameter	Dose Group (ppm)							
	0		250		3,500		7,500/12,000	
Males								
Stains, yellow, PGA	6		9		5		16	*
Stains, brown #	1		5		2		9	*
Body, scaly	6		5		7		13	
Females								
Stains, yellow, PGA	9		9		13		31	*
Stains, brown #	0		1		6	*	23	*
Body, scaly	1		0		3		15	*

* = $p < 0.05$; # includes tail, perigenital area (PGA) and perianal locations

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Table 5.5.2-2: Mean body weights (g) and cumulative body weight gain (g)

Parameter	Dose Group (ppm)							
	0		250		3,500		7,500/12,000	
Males								
Initial body weight	206.5		204.8		205.3		204.7	
Final body weight week 103	629.4		596.7		619.5		564.9	*
Body weight gain week 2	43.0		42.9		43.1		38.7	*
Body weight gain week 13	226.6		228.0		224.3		204.2	*
Body weight gain week 26	286.4		289.3		286.7		263.3	*
Body weight gain week 54	367.0		371.6		363.1		334.0	*
Body weight gain week 78	426.9		419.3		424.4		380.8	*
Body weight gain week 103	422.0		399.9		414.9		361.2	*
Females								
Initial body weight	152.0		151.1		152.2		151.6	
Final body weight week 103	386.6		393.3		398.0		331.8	*
Body weight gain week 2	18.8		17.9		17.5		15.6	*
Body weight gain week 13	91.2		86.4		87.1		78.9	*
Body weight gain week 26	116.0		109.6		111.2		99.0	*
Body weight gain week 54	176.9		156.1	*	163.0		134.2	*
Body weight gain week 78	231.3		218.5		231.1		171.9	*
Body weight gain week 103	233.9		241.1		246.8		180.5	*

* = $p < 0.05$

Hematology: Leukocyte differential counts and erythrocyte morphology determined at approximately 12, 18, and 24 months into the study provided no indication of a BYI 08330-induced change in either sex at any dose tested.

Gross necropsy, organ weights: Gross necropsy showed an increased incidence of focal to multifocal (1-4 mm) discoloured zones (sometimes raised) of the lung in 12,000-ppm females (1, 1, 3, 32*) which were attributed to compound administration. Relative lung weight was slightly increased in the 7,500 ppm male and the 12,000 ppm female group. The lung weight changes in 12,000 ppm females correlated with increased focal to multifocal discolorations observed in the lungs at necropsy. Absolute kidney weights were decreased in both sexes at 3,500 and 7,500/12,000 ppm without associated changes in relative kidney weights.

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In the kidney, relative weights were not clearly consistent with the absolute weight effect in both sexes at 3,500 and 7,500/12,000 ppm, which would suggest that the absolute changes were not a significant biological effect, but more likely a secondary response to decreased body weight gain. These changes were associated with microscopic findings noted in the same groups; however, a clear cause and effect of the microscopic finding in terms of decreased tissue weight cannot be made. Any other apparent change in absolute kidney weight (e.g., 250 ppm males) was considered to be incidental, due to the highly variable kidney weight data (particularly in males) and the lack of microscopic evidence of a direct toxicological insult by the test substance on the kidney that would account for a decrease in kidney weight. All other apparent organ weight changes were attributable to changes in the body weight profile.

Table 5.5.2-3: Mean absolute (g) and relative (%) organ weights

Parameter	Dose Group (ppm)							
	0		250		3,500		7,500/12,000	
Males								
Final body weight	620.6		600.6		614.8		566.6	*
Liver weight, absolute	19.396		18.551		19.219		18.437	
Liver weight, relative	3.119		3.104		3.133		3.274	
Lung weight, absolute	2.245		2.362		2.290		2.420	
Lung weight, relative	0.366		0.402		0.377		0.435	*
Kidney weight, absolute	4.329		3.910	*	3.886	*	3.697	*
Kidney weight, relative	0.709		0.659		0.635		0.662	
Females								
Final body weight	377.0		395.4		396.5		328.2	*
Liver weight, absolute	12.621		12.907		12.777		12.360	
Liver weight, relative	3.379		3.295		3.245		3.779	*
Lung weight, absolute	1.949		1.899		1.852		1.934	
Lung weight, relative	0.527		0.492		0.479		0.600	*
Kidney weight, absolute	2.979		2.851		2.658	*	2.572	*
Kidney weight, relative	0.820		0.741		0.691	*	0.796	

* = $p < 0.05$

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Histopathology:

Non-neoplastic changes: Statistically significant changes in the incidence of non-neoplastic lesions were detected in the lung, kidneys, testis/epididymis and liver.

Lung: An increased incidence of alveolar macrophage accumulation (M: 7,17*,17*,3; F: 20,14,18,3), a common background lesion in the rat, and a complex of changes described as interstitial pneumonia (M: 22,15,19,44*; F: 4,13*,14*,52*) was noted in the lungs of 7,500-ppm males, and 12,000-ppm females. Both findings were attributable to exposure to the test substance and were interpreted as a continuum of morphologic changes (correlating with the gross lung observations noted in 12,000 ppm females) and a such were evaluated together (M: 29, 32, 36, 47*; F: 24, 27, 32, 55*). Macrophage accumulation without additional change was coded as such. Interstitial pneumonia was diagnosed with the presence of one or more of the following changes: presence of lymphocytes, cholesterol clefts, interstitial thickening of the alveolar septa by connective tissue, or increased alveolar pneumocytes and presence of occasional extravasation of erythrocytes (micro hemorrhage). The lung effects described above were focal to multifocal in distribution and involved a small overall portion of the lung tissue triggering no respiratory symptoms.

Kidney: An increased incidence of tubular dilatation was observed in the outer medulla (presumed to be in the loop of Henle) in both sexes of the 3,500 ppm groups and in the 7,500/12,000 ppm groups. The affected renal tubules were dilated and were either empty or contained pale eosinophilic proteinacious material, usually granular. Negligible cellular death, regeneration, or inflammatory responses to the tubular dilatation was present. In addition, there were no associated tubular casts in more distal tubular locations such as the collecting ducts. This lesion in the 3,500 and 7,500/12,000 ppm animals was considered treatment-related and was presumed to be reflected in the decreased kidney weights although there was no associated loss of tissue. As such, a clear relationship between this finding and decreased tissue weight cannot be made. Moreover, the high background incidence of chronic nephropathy greatly influenced the variability of the kidney weights measured in this study which is commonly noted in 2-year rat studies.

Testis: In males, there was a background incidence of typical aging changes including variably severe testicular degeneration, oftentimes noted grossly as soft, brown, or reduced in size (12,8,10,6). In addition to this change in the 7,500-ppm males, there were a few animals with generally slight morphologic testicular change, more subtle than distinct tubular degeneration, characterized by a depletion, asynchrony, and degeneration of the latter stage spermatids (0,0,0,9*).

Epididymis: An increased incidence of immature/exfoliated germ cells/debris was observed in the lumen contents of the head, body, and tail of the epididymides at 7,500 ppm (6,10,6,31*), which correlates with the testicular change. There was no morphologic change noted in the anatomical structure of the epididymis tissue in conjunction with the luminal content of exfoliated immature / abnormal testicular stages.

Liver: In the bile duct, a nonstatistical increased incidence of hyperplasia/fibrosis with associated minimal periportal mononuclear cell infiltrate was noted in 12,000-ppm females.

Neoplastic changes:

Type, incidence and organ distribution of all neoplastic lesions was not significantly different between treated and control rats.

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Table 5.5.2-4: Average incidence and severity (figures in parenthesis) of microscopic findings

Parameter	Dose Group (ppm)							
	0		250		3,500		7,500/12,000	
Males								
Lung, macrophages, alveolar	7 (1.3)		17 (1.2)	*	17 (1.4)	*	3 (1.0)	
Lung, pneumonia, interstitial	22 (1.6)		15 (1.7)		19 (1.7)	*	44 (2.3)	*
Combined	29		32		36		47	*
Kidney, dilatation, tubular [a]	0		0		8 (1.8)	*	19 (1.9)	*
Kidney, nephropathy, chronic	44 (2.3)		47 (1.7)		43 (2.0)		42 (1.8)	
Testis, degeneration	12 (3.4)		8 (3.5)		10 (2.9		6 (3.2)	
Testis, spermatid degeneration, depletion, asynchrony	0		0		0		9 (1.2)	*
Epididymis, germ cell, exfoliated, debris	6 (2.2)		10 (1.9)		6 (1.8)		31 (2.5)	*
Liver, biliary hyperplasia /fibrosis	14 (1.3)		1 (1.0)		0		9 (1.3)	
Females								
Lung, macrophages, alveolar	20 (1.3)		14 (1.1)		18 (1.1)		3 (1.7)	
Lung, pneumonia, interstitial	4 (1.5)		13 (1.4)	*	14 (2.1)	*	52 (3.4)	*
Combined	24		27		32		55	*
Kidney, dilatation, tubular [a]	0		0		16 (1.8)	*	42 (2.5)	*
Kidney, nephropathy, chronic	31 (1.9)		29 (1.4)		22 (1.3)		22 (1.6)	
Liver, biliary hyperplasia /fibrosis	18 (1.9)		24 (1.7)		24 (1.8)		32 (1.7)	

* = $p < 0.05$; [a] thin (descending) limb of the loop of Henle

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Conclusions: The chronic (24 months) NOAEL was established at 250 ppm (equal to 12.5 mg/kg bw/day) in male rats, based on decreased absolute kidney weights and an increased incidence of renal tubular dilatation at 3,500 ppm (equal to 169 mg/kg bw/day).
There was a slightly increased incidence of testicular tubular degeneration and immature/exfoliated germ cells in the epididymal lumen in 7,500 ppm male rats after two years of treatment (equal to 373 mg/kg bw/day).
The chronic (24 months) NOAEL in females was established at 250 ppm (equal to 16.8 mg/kg bw/day), based on decreased absolute kidney weights and an increased incidence of renal tubular dilatation at 3,500 ppm (equal to 229 mg/kg bw/day).
Based on type, incidence and organ distribution of neoplastic lesions in treated and control rats, there was no indication for an oncogenic effect of BYI 08330.

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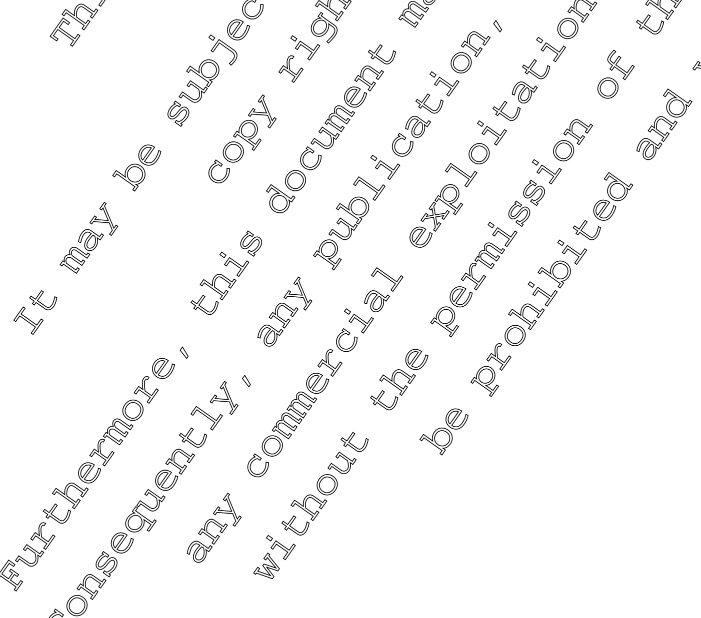
IIA 5.5.3 Carcinogenicity study in the mouse

Report:	**KIIA 5.5.3/01, [redacted]; 2005**
Title:	Technical Grade BYI 08330 (Common Name Spirotetramat): An Oncogenicity Testing Study in the Mouse
Report No & Document No	201359-1 M-275506-02-1
Guidelines:	OPPTS Guideline No. 870.4200 (1998); OECD Guideline No. 451 (1981); JMAFF 12-Nousan-No. 8147; Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory). Deviations: none

Executive Summary

In a chronic (18-months) feeding study, BYI 08330, batch no.: 08045/0014, purity: 97.4 – 98.5 %, was administered continuously for approximately 18 months to 55 male and 55 female CD-1 mice (CD-1 [ICR]/BR)) at nominal dietary concentrations of 0, 70, 1,700, or 7,000/6,000 ppm. The high-dose level for this study was initiated with a dietary concentration of 7,000 ppm. Starting with week 12 of the study, this level was reduced to 6,000 ppm in order to achieve an average active ingredient intake of approximately 1,000 mg/kg/day through 18 months of exposure. The dietary concentrations were equal to doses of 0, 10.9, 263, and 1022 mg/kg bw/day for males and 0, 13.7, 331, and 1319 mg/kg bw/day for females.
No effects of any kind emerged in the mouse, which was tested up to the limit dose in this 18-months feeding study.
Based on type, incidence and organ distribution of neoplastic lesions in treated and control mice, there was no indication for an oncogenic effect of BYI 08330.
The NOAEL was 7,000/6,000 ppm (equal to 1022 or 1319 mg/kg bw/day in males or females, respectively), based on the absence of compound-induced toxicological responses.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I. Material and methods

A. Materials:

1. Test Material: 3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro [4.5]dec-3-en-4-yl ethyl carbonate (BYI 08330; common name spirotetramat)

Description:	Technical grade, white powder
Lot/Batch #:	08045/0014
Purity:	97.5% (7/2003); 97.8% (12/2003); 97.6% (6/2004); 98.5% (1/2005); 97.4% (5/2005)
Compound Stability:	Stable at room temperature (~ 22 °C)
CAS # of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1

2. Vehicle and/or positive control: None

3. Test animals:

Species:	Mouse; male and female (nulliparous and non-pregnant)
Strain:	CD-1; (CD-1 [ICR]/BR)
Age/weight at study initiation:	Approximately 8 weeks old / 30.6 - 30.8g mean group weight males; 24.4 - 25.1g mean group weight females
Source:	[redacted]
Housing:	Housed in suspended stainless steel wire-mesh cages, each containing a feeder, a source of water (pressure-activated water nipples), and deotized (sanitized) cage board in the bedding tray. Animals passing the initial shipment exam were co-housed during a portion of the Acclimatisation and later housed individually (for the remainder of the Acclimatisation and throughout the exposure period). Cage racks were rotated monthly.
Diet:	[redacted] Certified Rodent Diet 5002 in "meal" form; *ad libitum*
Water:	Municipal water supply of Kansas City, MO; *ad libitum*.
Environmental conditions:	**Temperature:** 18 to 26 °C **Humidity:** 30 to 70% (relative); high range relative humidity was 71% on Days -10 (pre-exposure) and 39, and 73% on Day 11. **Air changes:** 13.58 changes/hr (minimum daily average) **Photoperiod:** 12 hr of light [7:00 a.m. to 7:00 p.m., or 8:00 a.m. to 8:00 p.m.] alternating with 12 hr of darkness. Deviations in the light/dark cycle occurred 3 times (2 planned; 1 unplanned) over the course of the study. The light cycle is unknown for Days 202 (Edstrom version 5 installed) and 234 (power outage occurred, smart box replaced due to lightning strike). None of the deviations was of a duration as to impact study outcome.
Acclimatisation:	10/20/03 (receipt) - 10/27/03 (release for study)

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B. Study design:

1. In-life dates: Start: 10/27/03 (released for study); 10/28/03 (study initiation); 11/03/03 (initiation of exposure)
End: 05/11/05 (terminal sacrifice complete)

2. Animal Assignment/Dose Levels: 10/28/03; assigned to a control or one of three chemically-treated groups (as noted in Table 1) using a weight stratification-based computer program obtained from INSTEM Computer Systems (Stone, Staffordshire, UK). Each animal on study identified with a microchip (, DE) subcutaneously implanted on their dorsal surface in the region between the scapulae; the chip was encoded with a unique number, specifying the animal's sex, dose group, cage number, and study affiliation.

Test Group	Conc. in Diet (ppm)	Dose to animal (mg/kg; 0±Std)	Main Study 18 months	
			Male	Female
Control	0	0	55	55
Low (LDT)	70	m: 10.9±1.4 f: 13.7±2.2	55	55
Mid (MDT)	1,700	m: 263±35 f: 331±49	55	55
High (HDT)	7,000 (1)	m: 1022±196 f: 1319±290	55	55

(1) The high-dose level for this study was initiated with a dietary concentration of 7,000 ppm. Starting with Week 12 of the study, this level was reduced to 6,000 ppm in order to achieve an average active ingredient intake of approximately 1,000 mg/kg/day through 18 months of exposure.

3. Dose selection rationale:

Doses were based principally upon the lack of a toxicological response in the mouse over the course of a subchronic toxicity testing study at doses up to and including 7,000 ppm (Report No. 201284). It was anticipated that the low and high doses chosen of 70 ppm and 7,000 would constitute a *no-observed-adverse-effect level* and a *limit dose*, respectively, with the intermediate dosage of 1,700 ppm serving to confirm any dose response relationships that may have emerged.

The probable routes of human exposure to BYI 08330 are via ingestion of foodstuffs that might contain low residues of the test substance, accidental ingestion, or dermal contact during manufacture or use. Thus, formulation with the feed was an appropriate route of administration with which to develop the toxicological profile of the test substance in a given test species.

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4. Diet preparation and analysis:

The test compound was mixed directly with the feed; control diet consisted of untreated feed. Samples of each batch of feed mixed were 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 and stored under freezer conditions until presented to the animals the following week.

BYI 08330 was administered at a constant concentration in the feed for the duration of the study. The concentration of the AI of the test substance in the control, 70-ppm, and 1,700-ppm test diets was analytically verified from samples taken during Weeks 1, 2, 3, 8, 12, 16, 20, 24, 28, 32, 37, 41, 46, 50, 54, 58, 63, 67, 71, and 76. The concentration of the AI of the test substance was analytically verified from samples of the 7,000-ppm test diet taken during Weeks 1, 2, 3, and 8, and from samples of the 6,000-ppm test diet taken during Weeks 12, 16, 20, 24, 28, 32, 37, 41, 46, 50, 54, 58, 63, 67, 71, and 76. In addition, one recovery sample was analyzed with each group of samples during the test concentration analyses. The homogeneity and stability of BYI 08330 when mixed in the dietary carrier was characterized prior to the in-life phase of the study and again just prior to termination of the in-life phase of the study.

To evaluate the uniformity of distribution/homogeneity of the test substance in the diet, an analysis of feed containing nominal concentrations of 70 and 12,000 ppm BYI 08330 was conducted. Three (3) samples were taken for analysis from each of 3 distinct layers (top, middle, bottom; 9 samples total) in a Hobart mixing bowl. A homogeneous distribution of the test substance in the feed was defined in terms of a percent relative standard deviation (%RSD, same as coefficient of variation; CV), derived from the 9 samples taken, which was ≤ 10%. Homogeneity was again analyzed, just prior to termination, using 1 sample from the top, middle, and bottom mixing bowls containing nominal concentrations of 70 and 6,000 ppm BYI 08330.

To evaluate the stability of the test substance in the diet, a time and temperature analysis of feed containing nominal concentrations of 70 and 12,000 ppm BYI 08330 was conducted. Stability in the feed was assessed following 0 and 7 days of room temperature storage (~22 ºC) and 7, 14, and 29, and 32 days of freezer storage (~ -23 ºC; 12,000 ppm evaluated at 29 days; 70 ppm evaluated at 32 days). The stability analysis was carried out as follows: An initial sample was taken immediately after the test substance was mixed with the ration. Each batch of ration, mixed at the 2 concentrations, was then divided into 2 portions. One portion was placed in a room temperature environment, the other in the freezer. Feed samples which remained at room temperature were sampled for analysis on Days 0, 1, and 7; feed samples which remained in the freezer were sampled for analysis on Days 7, 14, 29 (12,000 ppm only), and 32 (70 ppm only). Chemical stability in the ration was defined in terms of that period of time that 80% of the test substance, relative to the initial concentration, was recoverable.

The liquid chromatographic methodology used to perform the dietary analyses required for this study (i.e., homogeneity, stability, and concentration verifications) has been described previously.

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Results:

Homogeneity Analysis: The mean concentrations of BYI 08330 in the feed, sampled from 3 distinct layers in the mixing bowl and containing a nominal concentration of either 70 or 12,000 ppm, were determined to be 68.9 ppm (range 64.9-73.2 ppm; %RSD = 3.8%) and 12,157 ppm (range 11,844-12,567 ppm; %RSD = 2.1%), respectively. Based on a %RSD ≤ to 10%, BYI 08330 was judged to be homogeneously distributed in the feed over a concentration range of 70-12,000 ppm. Homogeneity analysis conducted just prior to study termination again confirmed homogeneity of the test substance in rodent ration. The mean concentrations of BYI 08330 in the feed, sampled from 3 distinct layers in the mixing bowl and containing a nominal concentration of either 70 or 6,000 ppm, were determined to be 65.8 ppm (range 61.9-70.1 ppm; %RSD = 6.3%) and 5,951 ppm (range 5,923-5,993 ppm; %RSD = 0.6%), respectively.

Stability Analysis: Following 7 days of room temperature storage, the analytically-determined concentration of the AI of the test substance in the 70- or 12,000-ppm admixture was determined to be 69.8 ppm (68.9 ppm on Day 0) and 11,948 ppm (12,157 ppm on Day 0), respectively. Following 29 and 32 days of freezer storage, the analytically determined concentration of the AI of the test substances in the 12,000- and 70-ppm admixtures was determined to be 12,474 and 68.0 ppm, respectively (12,157 and 68.9 ppm, respectively on Day 0). BYI 08330 mixed in rodent ration was judged to be stable at room temperature for at least 7 days and following freezer storage for a minimum 29 days, over a concentration range of 70-12,000 ppm.

Concentration Analysis: Mean analytical concentrations for each dose group were 67.7, 1,655, 5,986, and 6,873 ppm, ranging from 97-100% of the corresponding nominal concentrations of 70, 1,700, 6,000, and 7,000 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 97% and ranged from 89-103% for rodent ration spiked with 70, 1,700, 6,000, and 7,000 ppm BYI 08330.

5. Statistics:
Continuous data that were examined statistically were evaluated initially for equality or homogeneity of variance using Bartlett's test (, 1967). Group means were further analyzed by a one-way variance analysis (ANOVA) (, 1967) followed by Dunnett's test (Dunnett, 1955, 1964). Frequency data were initially examined for trends; data suggestive of a potential effect were then statistically evaluated using the chi-square, Fisher exact, or chi-square and Fisher exact tests. On a case by case basis, and at the discretion of the study director, data were subject to additional statistical procedures other than those mentioned above. For the Bartlett test, a probability (p) value ≤ 0.001 was considered significant; for all other statistical tests, differences with p values ≤ 0.05 were considered statistically significant. All statistical evaluations were performed using software obtained from either INSTEM Computer Systems or SAS Institute Inc. (Cary, NC).

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C. Methods:

1. Observations:

1a. Cageside Observations:

For approximately 79 weeks of the study, a general assessment of all remaining animals was carried out at least daily (generally twice daily during the normal work week and once daily on weekends and holidays). These assessments were generally with respect to moribundity and mortality, but also included a more general clinical observation for readily apparent clinical signs (in addition to moribundity and mortality).

1b. Clinical Examinations:

Once each week for approximately 79 weeks of the study, detailed physical examinations for clinical signs of toxicity were performed on all surviving animals. These examinations included evaluation of external surface areas, orifices, posture, general behavior, respiration, and excretory products.

2. Body weight:

Individual body weight determinations were carried out weekly for the first 13 weeks of the study and every 4 ± 1 weeks thereafter on all surviving animals. Body weights were also measured immediately prior to all necropsies to allow for calculation of organ to body weight ratios.

3. Food consumption and compound intake:

Individual food consumption determinations were carried out weekly for the first 13 weeks of the study and every 4 ± 1 weeks thereafter on all surviving animals. In addition, using specifically defined criteria, food consumption data were corrected, as conditions dictated, to account for misleading indications of food intake (i.e., excessive spillage, clogged feeders, etc.). Test substance intake (mg/kg body wt/day) was calculated using weekly mean 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

4. Ophthalmologic examination:

Ophthalmologic examinations were not conducted.

5. Hematology & Clinical Chemistry:

Blood was collected at approximately 12 and 18 months into the study from all surviving animals for differential determinations with the exception of animal RM3155 at 12 months. In all cases, blood was sampled via the orbital sinus from non-fasted animals. Differential blood counts were determined on all animals sacrificed due to moribundity with the following exceptions: RM1105, RM1131, RM2006, and RM2051. Clinical Chemistry determinations were not performed.

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5a. Hematology

	Hematocrit (Hct)	x	Leukocyte differential count*
	Hemoglobin (Hgb)		Mean corpuscular Hgb (MCH)
	Leukocyte count (WBC)		Mean corpuscular Hgb concentration (MCHC)
	Erythrocyte count (RBC)		Mean corpuscular volume (MCV)
	Platelet count (PLTS)		Reticulocyte count (Retic)
	Blood clotting measurements	x	Erythrocyte morphology
	(Thromboplastin time)		Heinz bodies (HZ)
	(Clotting time)		
	Prothrombin time (PT)		

* Minimum required for carcinogenicity studies (Cont. and HDT unless effects are observed) based on Guideline 870.4200 & OECD 451.

The CHECKED (X) parameters were examined.

6. Urinalysis:

Urinalysis determinations were not performed.

7. Sacrifice and Pathology:

7a. Gross pathology:

A complete postmortem examination was conducted on all animals (surviving, found dead, sacrificed *in extremis*) which included (1) documenting and saving all gross lesions, (2) weighing designated organs, and (3) collecting representative tissue specimens for histopathologic evaluation. Animals surviving at termination were sacrificed by CO_2 asphyxiation. Tissues were preserved and organ/body weight ratios were calculated for weighed organs.

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	DIGESTIVE SYSTEM		**CARDIOVASC./HEMAT**		**NEUROLOGIC**
	Bile duct* (rat)	X	Aorta*	XX	Brain*+
X	Cecum*	X	Bone marrow*	X	Cerebellum
X	Colon*	XX	Heart*+	X	Cerebrum-Midbrain
X	Duodenum*	X	Lymph node, cervical*	X	Medulla/Pons
X	Esophagus*	X	Lymph node, mesenteric*	X	Eyes*
X	Gall bladder* (not rat)	XX	Spleen*+	X	Nerve, optic*
X	Ileum*	X	Thymus	X	Nerve, sciatic*
X	Jejunum*			X	Pituitary*
XX	Liver*+		**UROGENITAL**	X	Spinal cord, cervical*
X	Pancreas*	X	Cervix	X	Spinal cord, lumbar*
X	Rectum*	X	Clitoral gland^	X	Spinal cord, thoracic*
X	Salivary glands*	X	Epididymides*+		
X	Stomach, glandular*	XX	Kidneys*+		**OTHER**
X	Stomach, non-glandular*	X	Mammary gland*	X	Bone, femur
X	Tongue	XX	Ovary*+	X	Bone, rib/cc jct
X	Tooth	X	Preputial gland^	X	Bone, sternum
		X	Prostate*	X	Gross lesions and masses*
	RESPIRATORY	X	Seminal vesicle*	X	Harderian gland
X	Larynx*	XX	Testicle*+	X	Joint, fem/tib
XX	Lung*++	X	Urinary bladder*	X	Muscle, protocol
X	Nasal structure*	X	Uterus*+	X	Physical Identifier (ID chip) (1)
X	Nasopharynx*	X	Vagina^	X	Skin, protocol*
X	Oral structure			X	Zymbal's gland^
X	Trachea*		**GLANDULAR**		
		XX	Adrenal gland*+		
		X	Exorbital/lacrimal gland^		
		X	Parathyroid*		
		X	Thyroid*		

* Required for carcinogenicity studies based on Guideline 870.4200.

+Organ weight required in carcinogenicity studies.

++Organ weight required if inhalation route.

The CHECKED (X) tissues were collected for histological examination. The organs CHECKED (XX) were also weighed. The organs CHECKED (^) were preserved for possible micropathologic evaluation only.

(1) Starting on 04/15/05 (nominal Day 529), indelible tail marks (animal number) were used in lieu of replacing defective or "non-reading" ID chips. In such cases, the tail was collected.

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7b. Micropathology:
With the exception of the physical identifier (microchip and/or tail), the vagina, and the exorbital/lacrimal, clitoral, preputial, and Zymbal glands (of which the latter 5 were taken and preserved for possible micropathologic evaluation), representative sections of the tissues shown in Table 3 were processed for all control (0 ppm) and high-dose (7,000 ppm) animals, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under the light microscope. Tissues in which compound-related effects were detected were examined at lower doses as necessary to establish no-observed-effect levels. Target organs and gross lesions were examined for all animals. Histopathological lesions were assigned a semi-quantitative ranking of the severity grade/stage from "Normal (within normal limits)" to the "most severe case (Grade 5)," as described previously ([redacted], 1989; [redacted], 1990). The "grade number" was used to calculate an average grade for a particular observation within a dose group. Neoplastic lesions were generally not assigned a grade.
The initial micropathologic evaluation of this study was performed by [redacted] [redacted]. This evaluation was later reviewed by pathologists associated with Bayer CropScience LP, and included examination of various selected and randomly-selected slides.

II. Results

Findings:
General observations: Survival was unaffected by treatment, as the incidence of mortality was comparable between groups. Early deaths/survival numbers in males were 12/43, 13/42, 14/41, 11/44 in males and 19/36, 20/35, 14/41, 18/37 in females in ascending order of dose levels. Clinical observations attributable to exposure to the test substance were not observed in either sex at any dose tested. The general observation profile was consistent with that of the aging mouse. There were no treatment-related effects on body weight and food consumption in either sex at any dose tested.

Hematology: Evaluation of the data from blood collected at approximately 12 and 18 months into the study provided no indication of a BYI 08330-induced change in either sex at any dose tested. Determinations included leukocyte differential counts and erythrocyte morphology only.

Gross necropsy, organ weights: Evaluation of the general profile of absolute and relative organ weights as well as of gross lesions observed in this study did not indicate a BYI 08330-induced change in either sex at any dose tested.

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Histopathology:

<u>Non-neoplastic changes:</u>

Microscopic findings (non-neoplastic) attributable to exposure to the test substance were not observed in this study.

<u>Neoplastic changes:</u>

Microscopic findings (neoplastic) attributable to exposure to the test substance were not observed in this study.

Two incidences of tumors arising from brown fat were noted in high-dose males. Microscopically, the tumors were determined to be benign, and were composed of well-differentiated cells that resembled brown fat. However, there were occasional cells that appeared to originate from white fat. As such, the term “lipomatous neoplasm” was assigned to the lesion. In both cases, the tumors were noted grossly (1.5 to 2.0 cm in size) and were located in the interscapular region. Both animals lived until study termination. Similar tumors were not observed in females.

The report of this type of tumor is rare. One consideration that was made concerned the use of implantable identification transponders (ID chips), which were placed between the scapula (subcutaneously) prior to study. No direct evidence of involvement was documented in this study; however, implantable transponders (microchips) have been associated with the formation of various other tumor types in both mice and rats (Elcock *et al*., 2001: Tumors in long-term rat studies associated with microchip animal identification devices. Exp Toxic Pathol **52**, 483-491; Ghanta and Edmondson, 1990: Tissue reaction to an implantable identification device in mice. *Toxicol Pathol* **18** (3), 412-416.; Palmer *et al*., 1998: Fibrosarcomas associated with passive integrated transponder implants. *Toxicol Pathol* (meeting abstract) **26** (1), 170.). Any possible influence of the transponder (i.e., foreign-body tumorigenesis) under the circumstances described for this specific case is unclear. In this study, the data suggest that these tumors were spontaneous in nature. The incidence in the high dose was not significantly different from controls and no similar tumors were observed in any female animal. Both tumors were benign in nature and did not result in premature mortality in either case.

III. Conclusion

The NOAEL of the chronic feeding study in mice was established at 7,000/6,000 ppm (equal to 1022 or 1319 mg/kg bw/day in males or females, respectively), based on the absence of compound-induced toxicological responses.

Based on type, incidence and organ distribution of neoplastic lesions in treated and control mice, there was no indication for an oncogenic effect of BYI 08330.

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IIA 5.5.4 Mechanism of action and supporting data

Summary of mechanistic study results

Two mechanistic studies were conducted to identify time of onset and location of the first visible effects on the rat testis and to determine the causative metabolite of the testicular/sperm toxicity of BYI 08330.

Daily doses of 1,000 mg BYI 08330/kg bw/day were administered by gavage to male Wistar rats at different lengths of treatment periods with scheduled sacrifice time points after 3, 10, 21 or 41 days of treatment. Distinct clinical signs, reduced food consumption and body weight loss were noted during the study. Treatment-related death occurred in one animal at day 31. Morphological changes in the seminiferous germinal epithelium of the testis were evident at day 21 and day 41 of treatment and included degeneration / loss of elongating spermatids (steps 9-14) and degeneration of round spermatids (step 7-8). In the epididymis increased incidences of intraluminal aberrant cells were observed at day 21 and 41 associated with oligospermia at day 41. A slight increase in Sertoli cell vacuolation was noted at day 41. Histopathology of the prostate showed no treatment-related findings. Sperm analysis revealed abnormal epididymal spermatozoa after 21 and 41 days of treatment and a marked decrease in the numbers of spermatozoa at day 41.

The objective of the second mechanistic study with the BYI 08330-enol was to determine the causative agent of the testicular/sperm toxicity of BYI 08330. The structure of BYI 08330 consists of two parts; an enol entity and an acyl chain. The enol is the major metabolite of BYI 08330 in rats whereas the acyl chain of BYI 08330 aids the penetration of BYI 08330 into the plant and is cleaved once in the plant. As the structure of the acyl chain resembles that of the established testicular toxicant, methoxy acetic acid, it was proposed that the acyl chain may be responsible for the testicular toxicity of BYI 08330. Daily doses of 800 mg BYI 08330-enol/kg bw/day were administered by gavage to male Wistar rats for 21 days. The dose of 800 mg/kg/day BYI 08330-enol used in this study was chosen as it is equivalent to 1,000 mg/kg/day of the parent compound.

Based on the results of this second study, the effects are similar, both in terms of observations and magnitude of responses, to those recorded for the previous study (Report No. SA 04181) in which the parent compound (BYI 08330) was assessed. Distinct clinical signs, reduced food consumption and body weight loss was noted during the study; no mortalities were recorded. Morphological changes in the seminiferous germinal epithelium of the testis were evident after 21 days of treatment and included degeneration / loss of elongating spermatids (steps 9-14), diffuse sloughing of germ cells, degenerating round spermatids (step 7-8) and Sertoli cell vacuolation of minimal degree. In the epididymis exfoliated germ cells were noted. Sperm analysis revealed abnormal epididymal spermatozoa.

Thus, it is concluded, that the testicular/sperm toxicity of the BYI 08330 is unlikely to be due to the acyl chain of this compound but rather may be attributed to its major metabolite i.e., BYI 08330-enol.

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Mechanistic studies in rats

Report:	**KIIA 5.5.4/01, [redacted], 2005**
Title:	Evaluation of the potential reproductive toxicity in the male rat following daily oral administration by gavage
Report No & Document No	SA04181 M-252001-01-2
Guidelines:	No applicable guidelines
OECD/FIFRA GLPS	Yes (certified laboratory). Deviations: none

Executive Summary

The objective of this mechanistic study was to identify the primary target cell in male Wistar rat testis and epididymis and the time of onset of effects following daily oral administration of BYI 08330 at different lengths of treatment periods. BYI 08330, batch no.: 08045/0014, purity: 97.2 % was administered orally by gavage at a dose of 1,000 mg/kg/day to groups of eight male Wistar Hanover rats (strain: Crl:WI [Glx/BRL/Han] IGS BR) scheduled for sacrifice time points after 3, 10, 21 or 41 days of treatment. Another set of eight animals per group per scheduled sacrifice time point received the vehicle alone (an aqueous solution of methylcellulose 400 at 0.5%) and acted as control groups. Clinical signs were recorded daily, body weight and food consumption was measured at least weekly. A detailed physical examination was performed weekly throughout the study. Serial sacrifices were performed on days 3, 10, 21 and 41, approximately 4 hours after the last dosing. All animals were necropsied, selected organs weighed (testes, epididymis, prostate) and a range of tissues was taken, fixed and examined microscopically (left testes and epididymis, ventral prostate). In addition, following sacrifice, sperm from the right epididymis was collected for sperm enumeration and morphology assessment. Distinct clinical signs, reduced food consumption and body weight loss were noted during the study. Treatment-related death occurred in one animal at day 31. Morphological changes in the seminiferous germinal epithelium of the testis were evident at day 21 and day 41 of treatment and included degeneration / loss of elongating spermatids (steps 9-14) and degeneration of round spermatids (step 7-8). A slight increase in Sertoli cell vacuolation was noted at day 41. In the epididymis increased incidences of intraluminal aberrant cells were observed at day 21 and 41 associated with oligospermia at day 41. Histopathology of the prostate showed no treatment-related findings. Sperm analysis revealed abnormal epididymal spermatozoa after 21 and 41 days of treatment and a marked decrease in the numbers of spermatozoa at day 41.

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Results and Discussion:

General observations: Treatment-related death occurred in one animal on day 31. This animal showed increased salivation on days 17 and 21 and a slightly reduced body weight gain between days 21 and 28. For a limited time period after exposure, several of the remaining animals showed clinical signs of the skin/fur (lesion, piloerection, generalized or localized soiled fur), movement or behavior (reduced motor activity, hyper-reactivity, tremors), mouth (increased salivation), respiration (noisy, coughing) and general appearance (general pallor, wasted, hunched posture). A mean body weight loss was observed between days 1 and 10 and a static body weight between days 10 and 15. Mean terminal body weight was comparable to the control values at sacrifice on day 3, but was reduced by between 6 to 12% at sacrifice days 10, 21 and 41. Mean food consumption was statistically decreased by 22% from day 1 to 8, and slightly reduced by 6 to 10 % from day 8 to 22.

Table 5.5.4-1: Affected body weight

Parameter	Dose Group (mg/kg)			
	0		1,000	
Body weight day 1	**391**		**392**	
Body weight day 3	**399**		**391**	*
Body weight day 10	**417**		**388**	**
Body weight day 15	**428**		**395**	**
Body weight day 21	**442**		**406**	**
Body weight day 28	**453**		**404**	**
Body weight day 35	**466**		**411**	**
Body weight day 41	**478**		**421**	**
Overall body weight gain	**87**		**29**	**n.t.**

* = $p < 0.05$; ** = $p < 0.01$; n.t.=not tested

Sperm analysis: The absolute and relative numbers of spermatozoa after 41 days of treatment were markedly decreased by 77 and 68%, respectively, compared to controls. Other slight decreases of 6 to 15% in absolute and relative numbers of spermatozoa at days 3, 10 or 21, even if statistically significant, were attributed to individual variations or to a particularly high control value and were thus considered not to be treatment related.

After 41 days, the frequency of total abnormal spermatozoa was markedly increased in the treated group (72.0 % vs. 3.8 % in the control group). After 21 days, the frequency of total abnormal spermatozoa was slightly increased in the treated group (6.4% vs. 2.5% in the control group).

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Table 5.5.4-2: Sperm analysis

Control group								1,000 mg/kg							
Day 3		Day 10		Day 21		Day41		Day 3		Day 10		Day 21		Day 41	
Epididymal sperm count (absolute)															
184.4		218.4		199.4		214.3		160.6		188.5	*	169.6	*	50.3	**
Epididymal sperm count per gram of the caudal part of the epididymis															
849.6		940.6		871.5		878.1		801.7		818.0	*	808.0		281.4	**
Sperm morphology (Total abnormal cells in %)															
4.1		3.2		2.5		3.8		2.8		3.7		6.4	**	72.0	**

* = $p < 0.05$; ** = $p < 0.01$

Gross necropsy, organ weights: Gross pathology changes and organ weight differences (testes, epididymis) seen on day 3, 10 and 21 were considered to be incidental and not treatment-related. Gross necropsy on day 41 showed an increased incidence of small prostate in 2/7 animals, small and/or soft testes in 6/7 animals and small epididymis in 7/7 animals. On day 41, a treatment-related decrease in absolute and relative testes and epididymis weights was also observed.

Histopathology: No treatment-related microscopic findings were noted in the prostate at all sacrifice time points and in the testis and the epididymis on day 3 and day 10. Treatment-related changes were observed in the seminiferous tubules and in the epididymis after 21 and 41 days of treatment.
In the testis, marked degenerating elongating spermatids (steps 9 to 14 of the maturation cycle) were found in 8/8 treated animals, together with multinucleated giant spermatids in 2/8 rats and moderate to marked degenerating round spermatids in 4/8 rats. These morphological findings identified after 21 days of treatment are consistent with a treatment-related effect in round spermatids or late stage spermatocytes. In the epididymis, a slight to moderate increase of intraluminal abnormal aberrant cells were found in all treated animals after 21 days of treatment, as a consequence of degenerating spermatids observed in the testis. At final sacrifice on day 41, slight to severe degenerating elongating spermatids (steps 9 to 14) were found in 7/7 treated animals, together with loss of elongating spermatids (steps 9 to 19) in 5/7 treated rats. Multinucleated giant spermatids in 4/7 rats and marked degenerating round spermatids (around steps 7-8) in 5/7 rats were also observed. These morphological findings in the testes confirm what was observed after 21 days of treatment and may be consistent with a treatment-related effect in round spermatids. As a consequence of degenerating spermatids observed in the testis, a marked increase of intraluminal aberrant cells associated with a moderate to marked oligospermia were found in the epididymis in all treated animals on fay 41. Sertoli cell vacuolation was noted on day 41.

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Table 5.5.4-3: Incidence of treatment-related changes in the testis

Parameter	Day 21		Day 41	
Dose Group (mg/kg)	0	1,000	0	1,000
Number of animals examined	**8**	**8**	**8**	**7**
Degenerating round spermatids (step 7-8)				
moderate	**0**	**2**	**0**	**0**
marked	**0**	**2**	**0**	**5**
total	**0**	**4**	**0**	**5**
Multinucleated giant spermatids				
slight			**0**	**3**
moderate	**0**	**1**	**0**	**1**
marked	**0**	**1**	**0**	**0**
total	**0**	**2**	**0**	**4**
Degenerating elongating spermatids (step 9-14)				
slight	**0**	**0**	**0**	**1**
moderate	**0**	**0**	**0**	**1**
marked	**0**	**8**	**0**	**3**
severe	**0**	**0**	**0**	**2**
total	**0**	**8**	**0**	**7**
Loss of elongating spermatids (step 9-14)				
moderate	**0**	**0**	**0**	**2**
marked	**0**	**0**	**0**	**1**
severe	**0**	**0**	**0**	**2**
total	**0**	**0**	**0**	**5**
Sertoli cell vacuolation				
slight	**0**	**0**	**0**	**5**
moderate	**0**	**0**	**0**	**1**
total	**0**	**0**	**0**	**6**

In the epididymis, a slight to moderate (day 21) and marked increase in intraluminal aberrant cells (day 41) associated with a moderate to marked oligospermia (day 41 was found in all treated animals, which is considered a consequence of degenerating spermatids observed in the testis.

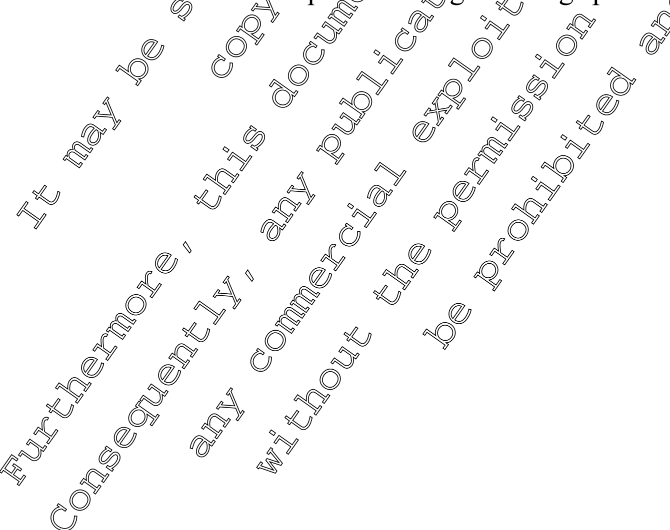

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Table 5.5.4-4: Incidence of treatment-related changes in the epididymis

Parameter	Day 21		Day 41	
Dose Group (mg/kg)	0	1,000	0	1,000
Number of animals examined	**8**	**8**	**8**	**7**
Increased intraluminal aberrant cell types				
slight	**0**	**2**	**0**	**0**
moderate	**0**	**6**	**0**	**0**
marked	**0**	**0**	**0**	**7**
total	**0**	**8**	**0**	**7**
Oligospermia				
moderate	**0**	**0**	**0**	**1**
marked	**0**	**0**	**0**	**6**
total	**0**	**0**	**0**	**7**

Conclusion: An investigative study using daily doses of 1,000 mg/kg BYI 08330 clarified the onset and location of adverse effects on germ cells in the rat testis. No effects on sperm count / morphology and no microscopic changes in the testes and epididymis were noted at day 3 and day 10. Subtle treatment-related changes in sperm parameters (abnormal cell morphology) were observed at day 21 associated with clear histopathological changes in the testes and in the epididymis (increase in intraluminal aberrant cells). Effects have been confirmed on day 41 of treatment. The main morphological change is a degeneration/loss of elongating spermatids (step 9 to 14) together with a degeneration of round spermatids (step 7 to 8) in the testis.

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Report:	**KIIA 5.5.4/02, ; 2006**
Title:	BYI 08330-enol: Investigation of the testicular/sperm toxicity in the rat following 21 days of exposure by gavage
Report No &	SA06011
Document No	M-273959-01-1
Guidelines:	No applicable guidelines
OECD/FIFRA GLPS	Yes. Deviations: none

Executive Summary

The objective of this mechanistic study was to determine the causative agent of the testicular/sperm toxicity of BYI 08330. In this study the metabolite BYI 08330-enol (batch number 692-101-09-0005: a beige powder, 96.8% purity), was assessed after oral administration by gavage at a dose level of 800 mg/kg/day for 21 days in five male Wistar Hanover rats Rj: WI (IOPS HAN). A similarly constituted group of 5 male rats received the vehicle alone (an aqueous solution of methylcellulose 400 at 0.5%) at the same dosage volume of 5 ml/kg/day and acted as a control. Clinical signs and body weights were recorded daily. Food consumption was measured weekly. A detailed physical examination was performed once during the acclimatization period and weekly throughout the study. All animals were sacrificed 4 hours after the last of twenty-one doses. All animals were necropsied and the testes and the epididymides were excised and weighed. Both testes and the left epididymis from each animal were fixed and examined microscopically. Sperm from the right epididymis were collected for sperm enumeration and morphological assessment.
Distinct clinical signs, reduced food consumption and body weight loss was noted during the study; no mortalities were recorded. Morphological changes in the seminiferous germinal epithelium of the testis were evident after 21 days of treatment and included degeneration / loss of elongating spermatids (steps 9-14), diffuse sloughing of germ cells, degenerating round spermatids (step 7-8) and Sertoli cell vacuolation of minimal degree. In the epididymis exfoliated germ cells were noted. Sperm analysis revealed abnormal epididymal spermatozoa.

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Results and Discussion:

General observations: No mortalities were recorded during the course of this study. A range of clinical signs were observed, particularly in the later stages of the study. These signs included localized soiled fur (anogenital region and mouth region; day 7-21), increased salivation (day 12-20) and reduced motor activity (day 20-21).

A mean body weight loss was observed on days 2, 14, 16, 18 and 20. The mean body weight gain on all other days of study was similar to control values. This resulted in the overall body weight gain being only 22% of the body weight gain of the controls. This was not, however, statistically significant due to the large standard deviations for both groups. The mean body weight was consistently, though not statistically significantly reduced compared to controls from 1.3% on study day 2 to 7% on the last day of dosing. The mean body weight was lower (-7%, not statistically significant) on day 21 when compared to controls.

Table 5.5.4-5: Affected body weight

Parameter	Dose Group (mg/kg)			
	0		1,000	
Body weight day 1	**454**		**455**	
Body weight day 3	**456**		**448**	
Body weight day 10	**471**		**388**	
Body weight day 15	**490**		**465**	
Body weight day 21	**500**		**465**	
Overall body weight gain	**46**		**10**	

* = $p < 0.05$; ** = $p < 0.01$

Mean food consumption was statistically reduced by 14.2% during study days 1-8 and by 8.5% during study days 8-15. The mean food consumption was similar to control levels during the last week of exposure.

Sperm analysis:

The absolute numbers of epididymal spermatozoa were slightly increased (5%, not statistically significant) after 21 days of treatment. However, the relative (to epididymal weight) numbers of spermatozoa were slightly decreased (7.9%, not statistically significant) after treatment. A high degree of interanimal variability was observed in both control and treated groups, particularly in the evaluation of the relative numbers (control range was 756-1109 x 10^6 spermatozoa/g tissue; treated range was 690 – 929 x 10^6 spermatozoa/g tissue).

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The frequency of total abnormal spermatozoa was significantly increased after 21 days of treatment with BYI 08330-enol when compared to control values (14.9% vs 3.2% in the control group). The majority of these abnormalities were isolated heads of normal morphology (60%), or spermatozoa with a normal head but possessing an abnormal mid-piece (33.3%). These observations were also recorded in a previous study investigating the parent compound.

Table 5.5.4-6: Sperm analysis

Control group		800 mg/kg	
Epididymal sperm count (absolute)			
189.8		199.7	
Epididymal sperm count per gram cauda			
918.7		846.1	
Sperm morphology (Total abnormal cells in %)			
3.2		14.9	*

* = $p < 0.05$;

Gross necropsy, organ weights: Gross pathology changes were considered to be incidental and not treatment-related. At 800 mg/kg, the mean relative testis weights were statistically significantly higher, but this change was mainly correlated to the lower terminal body weights.

Histopathology: A treatment-related change was found in the seminiferous tubules of both testes and in the left epididymis of each animal exposed to BYI 08330-enol after 21 days of treatment. Diffuse sloughing of germ cells associated with degenerating elongating spermatids (Step 9 to 14) were found in all treated animals. Multinucleated giant spermatids were observed in 2/5 rats and slight degenerating round spermatids (around step 7-8) in 1/5 rats. Sertoli cell vacuolation of minimal degree was also noted in one rat.

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Table 5.5.4-7: Incidence of treatment-related changes in the testis

Parameter	Day 21			
Dose Group (mg/kg)	0		800	
Number of animals examined	**5**		**5**	
Degenerating round spermatids (step 7-8)				
slight	**0**		**1**	
total	**0**		**1**	
Degenerating elongating spermatids (step 9-14)				
slight	**0**		**1**	
moderate	**0**		**4**	
total	**0**		**5**	
Multinucleated giant spermatids				
minimal	**0**		**1**	
slight	**0**		**1**	
total	**0**		**2**	
Sloughing of germ cells				
minimal	**0**		**2**	
slight	**0**		**3**	
total	**0**		**5**	
Sertoli cell vacuolation				
minimal	**0**		**1**	
total	**0**		**1**	

In the left epididymis, minimal to slight exfoliated germ cells were found in all treated animals, as a consequence of degenerating spermatids observed in the testes.

Table 5.5.4-8: Incidence of treatment-related changes in the epididymis

Parameter	Day 21			
Dose Group (mg/kg)	0		800	
Number of animals examined	**5**		**5**	
Exfoliated germ cells: focal/multifocal				
minimal	**0**		**1**	
slight	**0**		**4**	
total	**0**		**5**	

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Conclusion: Oral exposure of male rats to 800 mg/kg BYI 08330-enol for 21 days induced treatment-related effects in the testes and epididymis and induced an increase in spermatozoa with an aberrant morphology. The effects are similar, both in terms of observations and magnitude of responses, to those recorded for a previous study (Report No. SA 04181) in which the parent compound (BYI 08330) was assessed.
From the data generated in this study, it is concluded, therefore, that the testicular/sperm toxicity of the BYI 08330 is unlikely to be due to the acyl chain of this compound but rather may be attributed to its major metabolite i.e., BYI 08330-enol.

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IIA 5.6 Reproductive toxicity

Summary of reproductive toxicity

The reproductive toxic potential of BYI 08330 was tested in a multigeneration study in rats and in developmental toxicity studies in rats and rabbits. The observed effects on male reproductive function and performance triggered the conduct of several mechanistic studies and pharmacokinetic investigations. The developmental toxicity study in rats (main study) was supplemented by a second study (supplementary study) to further elucidate inconclusive findings of the main study.

Reproductive toxicity

A pilot one-generation study in rats was conducted with BYI 08330 at dietary concentrations of 0, 200, 500, 6,000 and 10,000 ppm. The study determined body weight gain declines (12%) in parental females of the 6,000 ppm group during lactation. No pregnancies and no implantation sites were observed in dams at the 10,000 ppm level. The lack of reproductive performance at 10,000 ppm (equal to 538 mg/kg bw/day) was caused by sperm cell abnormalities of P generation males. Sperm analysis revealed at 10,000 ppm increased numbers of abnormal sperm cells (amorphous sperm heads), accompanied by reduced epididymal sperm counts and a significant decline in both motility and progression of epididymal sperm cells. In addition, absolute and relative weight of the cauda epididymis was decreased in parental males at 10,000 ppm; testicular sperm counts were not affected. Histopathology showed abnormal sperm cells of minimal to moderate severity in the epididymis and the cauda epididymis.

At the 200-, 500-, and 6,000-ppm dietary levels, no treatment-related effects were observed on reproductive performance and function (mating-, fertility-, and gestation-indices, days to insemination, gestation length, median number of implants), on litter parameters determined at birth (litter size, percentage of males born, birth index, live birth index, viability index, lactation index), and on sperm parameters (morphology, motility, sperm count).

Neonatal effects at 6,000 ppm were limited to decreased body weights at lactation day 21 which corresponded with maternal body weight declines, recorded in 6,000-ppm females during lactation.

A subset of male F1 offsprings (n≈15/group) were exposed to the same dietary levels of BYI 08330 up to an age of eight to nine weeks and were thereafter maintained for an additional 5 to 6 weeks prior to performing organ weight measurements (testes, epididymis) and sperm analysis. In these F 1 interim males a significant decline in terminal body weight was observed at 6,000 ppm, together with slight effects on reproductive function. Sperm analysis indicated marginal declines in sperm motility and progression and a slight increase in abnormal sperm cells. Testicular and epididymal weights were not affected. Histopathology revealed abnormal sperm cells in the epididymides of a few animals (4/15) of the 6,000-ppm group.

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The occurrence of sperm effects in the 6,000-ppm F1 interim males is most likely caused by a higher dietary intake of BYI 08330 and a subsequently higher exposure to the test material when compared to the corresponding non-affected P generation males at 6,000 ppm which were exposed to average daily doses of 320 mg/kg bw/day. This is due to the fact that F1 interim males were [redacted]er at the start of dietary treatment when compared to P generation males and necessarily consumed more feed and chemical substance in relation to their body weight.

The two-generation study in rats was conducted with BYI 08330 at dietary concentrations of 0, 250, 1,000 and 6,000 ppm. The study revealed parental toxicity at 6,000 ppm. Effects included body weight gain declines in males of either generation, decreased terminal body weight, and increased renal multifocal tubular dilatation in F1-generation males. In females of both generations, reduced food consumption was noted at 6,000 ppm during lactation. In addition, F1-generation females of the 6,000 ppm group showed declines in body weights and in body weight gain at the end of the premating phase, decreased terminal body weights and an increase in multifocal renal tubular dilatation.

Up to the highest dietary concentration of 6,000 ppm, reproductive performance revealed no effect in either generation on any parameter measured (e.g., mating, fertility or gestation indices, days to insemination, gestation length, or the median number of implants). In addition, the litter parameters determined at birth (pup weight at birth, total number of pups born, stillborn pups, live birth index, percentages of male pups born, mean litter size at birth) were also unaffected in both generations up to 6,000 ppm. Histopathology of the reproductive tract (ovaries, oviducts, uterus, cervix, vagina) of females of either generation showed no treatment-related findings. Ovarian follicle counts (primordial follicles, antral follicles, or corpora lutea) of 6000-ppm F1-generation females showed no difference to controls. Female reproductive function (estrus cycle staging) was not affected in both the P- and F1-generations at any dietary level tested.

In P-generation males, reproductive function as measured by sperm count, motility, progression, and morphology as well as measured by weight, gross examination, and histopathology of reproductive organs remained unaffected at 6000 ppm (=419.3 mg/kg bw/day) and below.

In 6,000-ppm F1-generation males, sperm motility as well as epididymal and testicular sperm count was unchanged. However, abnormal sperm cells (8.9%) were still observed at 6,000 ppm (=486.7 mg/kg bw/day), but only one outlying male was affected to the extent that it compromised its fertilizing capabilities (i.e. 82% total abnormal sperm with 77% of these with amorphous heads). Histopathologically, abnormal epididymal sperm were only detected in this same male rat. No effects on reproductive function were observed in either the 1,000- or 250-ppm F1-generation males. Finally, micropathological evidence of the reversibility of these BYI 08330-induced sperm changes was provided in the subchronic rat study (Report No. 201136), which shared a top dose of 10,000 ppm with the P-generation pilot study.

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Neonatal toxicity at 6,000 ppm was limited to decreased body weights in F1 litters on lactation day 21 and in F2 litters on lactation days 14 and 21. Pup weights at birth were not affected. Pup weight declines corresponded with maternal body weight and food consumption declines. There were no treatment-related effects on the viability parameters (viability index, lactation index), and no clinical signs and no malformations were observed in both F1 and F2 pups. Gross necropsy, organ weights (brain, thymus, spleen, uterus) and histopathology of the reproductive organ system revealed no direct compound-related findings at any dietary levels tested. Maturation of external sexual organs (balano-preputial separation, vaginal opening) was not affected in F1 weanlings.

The NOAEL for parental toxicity in the two-generation reproduction study is 1,000 ppm (=70.7 mg/kg bw/day), based on declines in body weight and/or in body weight gain, reduced terminal body weights, reduced food consumption (females), and increased multifocal tubular dilatation in the kidneys at 6,000 ppm.
The NOEL for reproductive toxicity is 1,000 ppm (=79.5 mg/kg bw/day; F1 males). Marginal effects on reproductive function (abnormal sperm) occurred in the F1-generation males at the high dose level of 6,000 ppm, a dose level which elicits also parental toxicity; P-generation males were not affected. Reproductive performance was not affected at 6,000 ppm in either generation. The difference in outcome between 6,000 ppm P- generation males (no sperm effects) and 6,000 ppm F1-generation males (slight sperm effects) is most likely due to the higher dietary intake and subsequent higher exposure to the test material in F1-generation males (=486.7 mg/kg bw/day) when compared to P-generation males (=419.3 mg/kg bw/day).
The NOAEL for neonatal toxicity is 1,000 ppm, which is the same as for parental toxicity. It is based on slight growth retardation at 6,000 ppm corresponding with declines in maternal food consumption and body weight. Developmental landmarks were not affected.

In summary, BYI 08330 is of low potency regarding sperm cell toxicity and reproductive toxicity. Sperm analysis revealed an abrupt transition from no-adverse-effect-levels to adverse-effect-levels (steep dose-response curve) with reversible effects emerging only after high average daily doses of 538 and 487 mg/kg bw in the one-generation and two-generation reproduction studies, respectively. At the same time the two studies also established that daily exposures of 320 and 419 mg/kg bw in the one-generation and two-generation reproduction studies, respectively, failed to elicit effects on sperm cell number, morphology, and motility. In addition, one year of exposure to BYI 08330 at a rate of 414 mg/kg bw/day revealed no histopathological findings in either the testis or the epididymis of the rat (Report No. 201285). Following two years of steady exposure to the chemical at a rate of 373 mg/kg bw/day, an increased incidence of immature / exfoliated germ cells was identified in rat epididymis; a clear link to treatment is difficult to confirm, however, as the animals also presented confounding pathologic changes typical of aging rats (Report No. 201358).

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The observed effects on male reproductive function and performance triggered the conduct of several special studies designed to: 1) further characterize the action on spermatogenesis, 2) understand the nature of the steep dose-response and its emergence at higher doses, and 3) provide a greater understanding of the significance of these effects with respect to human risk assessment.
Two mechanistic studies (Report Nos. SA 04181 and SA 06011) were conducted to identify time of onset and location of the first visible effects on rat testis and to determine the causative metabolite of the testicular toxicity. The main points emerging from the mechanistic studies were:
1) the main morphological finding was a degeneration/loss of elongating spermatids (steps 9 to 14) together with a degeneration of round spermatids (steps 7 to 8);
2) the effects on sperm development in the testes were attributed to the enol (the major metabolite) of BYI 08330, and
3) morphological changes in the seminiferous germinal epithelium of the rat testis occur after 10 days of repeated high dose gavage treatment (1,000 mg/kg bw/day), suggesting the possibility of a toxicokinetic basis for the lesion.

Plasma and organ concentration ratios derived from toxicokinetic studies (Report Nos. MEF-06/328, and MEF-06/15) showed that BYI 08330 related residues in liver and kidney were markedly higher than in blood/plasma or other organs following a single low dose of 2 or 3 mg/kg body weight. This observation suggests the existence of saturable active transport processes in liver and kidney which have finite or limited capacities for the distribution / elimination of these BYI 08330 metabolites.
It is known that organic anion active transport systems are localized in the proximal tubule of the kidney and are particularly important in the renal uptake and excretion of anionic xenobiotics. Since urinary excretion is the predominant route of elimination of BYI 08330-metabolites, the anionic state of the enol and desmethyl enol at physiological pH is consistent with the postulate that active transport processes are involved in its renal elimination. Due to the anionic state of the BYI 08330-enol, the metabolite does not readily cross cell membranes and therefore has restricted distribution into tissues. The shift towards higher liver and kidney concentrations at a low dose indicates that active transport processes facilitate the influx of BYI 08330 metabolites into these organs against a concentration gradient. In contrast, a single high dose of 1,000 mg/kg revealed equally distributed concentrations in liver and kidney, when compared to plasma, indicating a saturation of active transport processes at higher doses, so that transport via diffusion becomes the predominant process for distribution into these tissues. It is possible that the transition from first-order to saturation kinetics, following repeated administration of BYI 08330, leads to a prolonged residence time of BYI 08330-metabolites in the body and/or increased concentrations in target organs.
This explains the observed abrupt transition from no-adverse-effect levels to adverse-effect levels (steep dose-response curve) in the reproductive toxicity studies in combination with the occurrence of sperm effects at a very high level of exposure (high dose phenomenon indicating low toxic potency).

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Physiology-based pharmacokinetic modelling (PBPK) were conducted to simulate the toxikokinetic behaviour of BYI 08330 (Report Nos. BTS-WSM0602 and BTS-WSM0603-1). The results demonstrated that the calculated tissue concentrations fit only to the experimentally derived liver-, kidney- and plasma-concentrations, if active transport processes for the uptake into organs and/or elimination from the body are assumed. Simulations also revealed that non-linear elimination kinetics – expressed as an over-dose-proportional increase in the AUC – are to be expected at dose levels above those being tested in the ADME (Absorption-Distribution-Metabolism-Excretion) study (>100 mg/kg bw). This prediction was supported by experimental data collected from a single dose organ depletion study (Report No. MEF-06/328), which revealed a delayed elimination of the enol from plasma after a single high dose of 1,000 mg/kg bw when compared to a low dose of 2 mg/kg bw (i.e. 24 hours after administration of 1,000 mg/kg bw, the plasma concentration was still 1/3 of the peak concentration measured 1 hour after administration, whereas a dose of 2 mg/kg bw was eliminated completely within 24 hours). Additional PBPK simulations, employing repeated administration of high doses of BYI 08330, revealed that at doses of 300 mg/kg and higher, plasma concentrations will rise more than dose-proportionally, leading to increased steady-state concentrations in plasma and organs after two weeks of daily treatment.

In conclusion, experimental data and PBPK simulation results support the hypothesis that active transport processes are involved in the uptake of BYI 08330 metabolites into liver and kidney and their subsequent renal elimination from the body. These processes become saturated at high doses of > 300 mg/kg bw, leading to an over-dose-proportional rise in plasma and tissue concentrations. This results in increased steady-state concentrations of BYI 08330 metabolites over time after repeated doses of > 300 mg/kg, finally exerting testicular toxicity in rats after 10 to 21 days of treatment.

The findings from an in vitro metabolism study suggest that it is likely that humans are less sensitive to BYI 08330-mediated testicular toxicity than rats. *In vitro* results from a Liverbead™ study using hepatocytes from male rats, mice, and humans (Report No. SA05319) revealed species differences in the metabolization of BYI 08330. Specifically, mouse and human hepatocytes, unlike the rat, were able to conjugate the enol via UDP-GT, thus reducing the systemic burden of the enol which has been shown to be the toxophore responsible for the degenerative testicular effects in the rat (Report No. SA 06011). This conjugation enables the utilization of separate active transport mechanisms in the kidneys and possibly in the liver (across the canalicular membrane into the bile). Moreover, the capacity of both the mouse and the human to conjugate the enol leads to a reduction in circulating free enol. Subsequently, the reduced concentration of free enol at the site of transport protein receptors in the kidneys will avoid a saturation of the elimination process, rendering these species less sensitive to BYI 08330-mediated testicular toxicity. This interpretation is further supported by the fact that the mouse showed no signs of testicular toxicity in any repeated dose study, which included exposures up to the limit dose of 1,000 mg/kg.

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As an overall conclusion from the reproductive and special studies, the data show that sperm cell toxicity in rats has been elicited in the BYI 08330 reproduction studies only after repeated high dose exposures to the test material (i.e. >300 mg/kg bw/day). The effects of BYI 08330 on rat sperm have been further characterized from a toxicological (degeneration of developing spermatids due to the enol metabolite of BYI 08330) and metabolic/toxicokinetic (saturation of active transport processes, species differences in glucuronide metabolism [rat ≠ mouse, human]) perspective, and provide support for the conclusion that these are high-dose phenomenons, with an increased sensitivity of the rat, and would not be encountered by humans even under the most extreme levels of anticipated prolonged and repeated exposure. Therefore, BYI 08330 is not considered to represent a reproductive hazard to humans at the expected low dose exposure scenarios routinely generated through the agricultural use of the chemical.
A separate position paper (Document number: M-297775-01-1) summarizes all relevant data characterizing the dose-response relationship for sperm cell toxicity of BYI 08330 in male rats and describing the likely reasons for the species specificity and for the abrupt transition from NOAELs to LOAELs following multiple (>10 daily) doses of >300 mg/kg bw/day.

Developmental toxicity

The developmental toxicity study in rats was conducted with doses of 0, 20, 140 and 1,000 mg/kg bw/day. A dose of 1,000 mg/kg bw/day caused increased incidences of skeletal variations (wavy ribs, 14th ribs, combined osseous and cartilaginous findings), increased incidence of retarded ossification, and a marginally increased incidence of common unspecific malformations (one case each of cleft palate, co-arctation of aortic arch, microphthalmia, and atrial septal defect of the heart; few cases of dysplastic forelimb bones, supernumerary lumbar vertebra, and altered appearance of sacral vertebral arches and pelvic shift). Additional historical control data have been provided in a position paper (Document number: M-296860-01-1) for forelimb bone dysplasia and sacral vertebral arch changes. Fetal and litter incidences of forelimb bone dysplasia at 1000 mg/kg bw/day ranged within the limits of historical control data. The finding of sacral vertebral arch alterations is not fully covered by previous study data, however, the attached figures of historical data show that the finding is appearing spontaneously in the rat strain used. Furthermore, an extensive pilot dose range finder study did not show this alteration at doses of 800 (n=4 litters) and 1,000 mg/kg bw (n=14 litters). All those increases were related to clear maternal toxicity (decreased feed intake, transient body weight loss, reduced body weight gain) observed at 1,000 mg/kg bw/day.
Although two of the malformations are rare observations (cleft palate, co-arctation of aortic arch), a specific potential of BYI 08330 to induce these types of malformations was not assumed since these findings occurred only once in the 1,000 mg/kg group, were different in type, and were as well seen spontaneously in control or low dose groups of recent developmental toxicity studies.
The increased incidences of wavy ribs and of retarded ossification of single bones at 20 and 140 mg/kg bw/day were considered an equivoval finding, requiring clarification via the conduct of a supplemental study.
Based on the clarifying results of this supplementary study, which revealed no indication for a treatment related increase of wavy ribs at doses of 10, 35 and 140 mg/kg bw/day and no dose-dependent changes on the degree of ossification, a treatment-related effect for the occurrence of wavy ribs and for retarded ossification in the main study at doses of 20 and 140 mg/kg bw/day was excluded.

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Taking into account the results of both developmental toxicity studies in rats, the maternal and developmental NOAEL was determined at 140 mg/kg bw/day based on reduced feed intake, transient body weight loss, reduced terminal body weight, and reduced corrected body weight gain in dams at 1,000 mg/kg bw/day and a marginally increased incidence in mainly common unspecific malformations, increased incidence of skeletal variations and increased incidence of retarded ossification in fetuses at 1,000 mg/kg bw/day. The study did not reveal a specific teratogenic potential of BYI 08330.

In the developmental toxicity study in rabbits, which was conducted with doses of 0, 10, 40 and 160 mg/kg bw/day the maternal NOAEL was 10 mg/kg bw/day; based on abortion in one animal at 40 mg/kg bw/day, most likely secondary to severe systemic maternal toxicity. A dose level of 160 mg/kg bw/day elicited severe systemic toxicity in conjunction with increased mortality and abortions. The NOAEL for embryo/fetal development was 40 mg/kg, based on a possibly increased incidence of fetuses with distinct liver lobulation at 160 mg/kg bw/day. The study did not reveal a specific teratogenic potential of BYI 08330.

Based on these results, a primary reproductive toxic or teratogenic potential of BYI 08330 was excluded in multigeneration studies in rats and in developmental toxicity studies in rats and rabbits. The observed high-dose effects on reproductive function and performance in rats represent no reproductive hazard to humans.

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IIA 5.6.1 Two generation reproductive toxicity in the rat

Report:	**KIIA 5.6.1/01, 2006**
Title:	Technical grade BYI 08330: A Dose Range-Finding Reproductive Toxicity Study in the Wistar Rat
Report No & Document No	201300-1 M-273578-02-1
Guidelines:	Whenever appropriate, this pilot study for a two-generation study complies with OECD 415, OECD 421, US-EPA-FIFRA §83-4, MAFF (Japan, 1985)
OECD/FIFRA GLPS	Yes (certified laboratory). Deviations: none

Executive Summary

This pilot study was conducted to select doses for a 2-generation study in rats. BYI 08330, batch no. 08045/0014, purity: 97.5 – 97.8%, was administered to groups of 10 male and 10 female Wistar Hanover rats (strain: Crl:WI(Glx/BRL/Han)IGS BR) at dietary concentrations of 0, 200, 500, 6,000 or 10,000 ppm. The dietary concentrations were equal to premating doses of 0, 10.5, 27.8, 320.1 and 537.9 mg/kg bw/day in males and to 0, 12.8, 31.4, 384.1 and 645.7 mg/kg bw/day in females, respectively. Parental (P) generation rats were approximately 14 weeks of age at the time of study initiation. They were pretreated for ten weeks, during the subsequent maximum one-week mating period and until the last litters were produced (males) or until lactation day 21 (females). Approximately half of the F1 offsprings were sacrificed at 21 days of age. A subset of F1 offsprings were maintained and observed for developmental landmarks and some males, referred to as F1 eight-to-nine week old interim animals, were maintained for an additional 5 to 6 weeks prior to performing organ weight measurements (testes, epididymis) and sperm motility (percent motile and percent progression), total sperm count (epididymal and testicular), and morphology.

The one-generation dose range finder study determined body weight gain declines in parental females of the 6,000 ppm group during lactation. Lack of pregnancies and the absence of implantation sites in dams at the 10,000 ppm level was caused by treatment-related effects on sperm cells of P generation males at 10,000 ppm (increased numbers of abnormal sperms, reduced epididymal sperm counts, decline in both motility and progression of epididymal sperm cells) accompanied by decreases in absolute and relative weight of the cauda epididymis. Testicular sperm counts were not affected at this dose level. Histopathology showed abnormal sperm cells of minimal to moderate severity in the epididymis and the cauda epididymis at 10,000 ppm.

Reproductive performance and function at the 200-, 500-, and 6,000-ppm dietary levels revealed no treatment-related effects on mating-, fertility-, or gestation-indices, days to insemination, gestation length, or the median number of implants. The litter parameters determined at birth (litter size, percentage of males born, birth index, live birth index, viability index, lactation index) as well as sperm (morphology, motility, and sperm count) were also not affected at the 200-, 500-, and 6,000-ppm dietary levels.

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Neonatal effects at 6,000 ppm were limited to decreased body weights (13.9%) and body weight gain declines (14.7%) at lactation day 21. Pup weight declines corresponded with maternal body weight declines.

In F1 eight-to-nine week old interim males significant declines in terminal body weight was observed at 6,000 ppm, together with slight effects on reproductive function. Sperm analysis showed marginal declines in sperm motility and progression and a slight increase in abnormal sperm cells. Histopathology revealed abnormal sperm cells in the epididymides in few animals (4/15) of the 6,000-ppm group. The occurrence of sperm effects in F1 interim males at 6,000 ppm is most likely caused by a higher dietary intake of BYI 08330 and a subsequent higher exposure to the test material when compared to the corresponding non-affected P generation males at 6,000 ppm which were exposed to average daily doses of 320 mg/kg bw/day.

The NOAEL for male parental toxicity is 6,000 ppm (equal to premating doses of 320 mg/kg bw/day), based on reduced cauda epididymal weights and effects on sperm at 10,000 ppm (equal to 538 mg/kg bw/day). The highest dietary exposure level (10,000 ppm) caused treatment-related effects on sperm cells of P generation males and included decreased sperm motility and progression, decline in epididymal sperm count and increase in abnormal sperm in the epididymis and the cauda epididymis.

The NOAEL for female parental toxicity in this pilot was established at 500 ppm (equal to <u>lactating doses</u> of 74.4 mg/kg bw/day), based on slight body weight gain declines during lactation in the P-generation females at 6000 ppm (equal to 831 mg/kg bw/day).

The NOAEL for male reproductive toxicity in this pilot study was established at 6,000 ppm in P-generation males (equal to premating doses of 320 mg/kg bw/day), based on decreased sperm motility and progression, decreased epididymal counts, increase in abnormal sperm, in the epididymis and the cauda epididymis in P-generation males at 10,000 ppm (equal to 538 mg/kg bw/day).

Other than the lack of pregnancies in the 10,000 ppm dose group, considered to be attributed to the effects on the sperm of the males, there were no reproductive effects observed in the females during the course of this pilot study.

The NOAEL for neonatal toxicity in this pilot study was established at 500 ppm, based on decreased body weights on lactation day 21 at 6,000 ppm. Pup weight declines corresponded with maternal body weight declines.

The NOAEL for male parental toxicity in this pilot study was established at 500 ppm in F1 eight-to-nine week old interim males, based on decreased terminal body weights at 6,000 ppm.

The NOAEL for male reproductive toxicity in this pilot study was established at 500 ppm in F1 eight-to-nine week old interim males based on decreased sperm motility and progression, increase in abnormal sperm in the epididymis and the cauda epididymis at 6,000 ppm. The effects in F1 eight-to-nine week old interim males were most likely caused by a higher dietary intake of BYI 08330 and subsequent higher exposure to the test material when compared to the corresponding non-affected P generation males at 6,000 ppm which were exposed to average daily doses of 320 mg/kg bw/day. This is due to the fact that F1 interim males were ███er at the start of dietary treatment when compared to P generation males and necessarily consumed more feed and chemical substance in relation to their body weight.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description: White powder

Lot/Batch No.: 08045/0014

Purity: 97.5% (7/2003), 97.8% (12/2003), 97.6% (6/2004)

Compound Stability: Stable at room temperature (~25±5°C)

Chemical Name: 3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate

CAS No. of TGAI: Non-stereo: 382608-10-8; cis: 203313-25-1

2. Vehicle and/or Positive Control: None.
Compound was dry mixed and administered through the diet.

3. Test Animals:

Species: Rat, males and females

Strain: Wistar Hanover rats Crl:WI(Glx/BRL/Han)IGS BR

Number of Animals: Total number ordered: 120 ; Number used on study: 50 male and 50 female.
Enough animals were ordered to ensure that a sufficient number of acceptable animals were available to conduct the study as designed.

Age at day zero of study: (P) 14 wks; (F_1) 14 wks

Wt. at day zero of study: (P) Males: 296.8 - 353.0 g; Females: 180.1 - 217.6 g

Source: [redacted]

Animal Receipt: Upon receipt, animals were examined by a veterinarian prior to release for study.

Housing: Animals passing the initial shipment exam were housed individually (except during the mating phase and as noted below for the F_1 pups) in suspended stainless steel cages and deotized cage board in the bedding trays. During gestation and lactation, individual dams (and litters) were housed in polycarbonate cages with corn-cob bedding.

Diet: *ad libitum* - [redacted] Chow 5002 meal

Water: *ad libitum* - pressure-activated water nipples

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Environmental conditions:	**Temperature:**	Room temperature 19° to 25°C
	Humidity:	Relative humidity 30 to 70%
	Air changes:	Minimum of 10 air changes per hour, based on average number of changes per hour per day
	Photoperiod:	Daily photoperiod of 12 hrs of light [6:00 a.m. to 6:00 p.m.] alternating with 12 hrs of darkness
Acclimatisation:	7 days	

All procedures associated with the animals were approved by the Institution's [redacted] and Use Committee (IACUC); and during the course of the study, room conditions were monitored and recorded in a consistent and timely manner. Feed and water samples were collected periodically and analyzed for potential contaminants; the concentrations outlined in the "Certification Profile" for [redacted] Chows ([redacted] International, 1998) were used as a standard of comparison for judging the acceptability of any contaminants detected in the analyzed feed samples.

B. Procedures and study design

1. Mating Procedure: Males and females were exposed to the test material for ten weeks prior to mating. Mating was accomplished by co-housing one female with one male for up to seven 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 which 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.

2. Study Schedule: Fifty female and fifty male rats were assigned to one of five treatment groups (10 animals/sex/group): nominal doses of 0, 200, 500, 6,000, and 10,000 ppm technical grade BYI 08330 in the diet. Animals were exposed to the treated feed throughout the entire in-life phase of the study. The parental animals (P-generation) were given test diets for 10 weeks prior to mating. Selection of F_1-pups to evaluate preputial separation and vaginal patency, as well as, sperm analysis was made when the pups were 21 days of age. The F_1 generation male pups were maintained until 8-9 weeks old and the females 5-6 weeks old prior to sacrifice.

3. Animal Assignment: Following a minimum of six days of acclimation and prior to the experimental start of the study, animals were examined by a veterinarian and released for study use. The animals were assigned to either a control or one of four chemically-treated groups using a weight stratification-based computer program (INSTEM Computer Systems, Stone, Staffordshire, UK). Only those animals falling within +/- 20% of the mean for all animals were put on study. Once animals were distributed into their dose groups, each rat on study had a microchip ([redacted], DE) subcutaneously implanted on their dorsal surface in the region between their scapulae. At the very least, the chip was encoded with a unique number, specifying the animal's

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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 which were born alive were identified by tattoo and pups found dead were identified with a marking pen. All animals not placed on study (for whatever reason) were ultimately sacrificed; however, prior to that time the animals were available, subject to the approval of the IACUC, for alternative uses which may have been unrelated to this study.

Test Group	Dose in Diet [a] (ppm)	Animals/group			
		P Males	P Females	F_1 Males Day 21/ Days 59-64	F_1 Females Day 21/ Days 37-42
Control	0	10	10	14/17	11/15
Low (LDT)	200	10	10	12/13	13/13
Mid 1 (MDT 1)	500	10	10	16/16	15/16
Mid 2 (MDT 2)	6,000	10	10	12/15	16/18
High (HDT)	10,000	10	10	0/0	0/0

a Diets were administered from beginning of the study until sacrifice.

4. Dose Selection Rationale: Doses were based principally upon the toxicological profile which emerged in the rat over the course of a subchronic toxicity testing study (Report No. 201136) and two developmental studies (Report Nos. AT01413 and AT01512). It was anticipated that by using such a broad range of doses that a more definitive toxicological profile would emerge in the animal that would allow the selection of appropriate doses at which to conduct a subsequent two-generation reproductive toxicity testing study in the rat.

5. Dosage Preparation and Analysis: The test compound was mixed directly 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 the same 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 every two weeks during the 10-week premating period and weekly (or at greater intervals depending on freezer stability) thereafter and stored under freezer conditions until presented to the animals the following week (or weeks). Additionally, the entire batch of replacement admixture for each treatment group was used for subsequent weekly feedings.

The mean daily intake of the test substance (mg BYI 08330/kg bw/day) throughout this one-generation reproduction study at nominal dietary concentrations of 0, 200, 500, 6,000 or 10,000 ppm, respectively, is summarized in the following table.

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Phase of Study	200 ppm in mg/kg/day[a]	500 ppm in mg/kg/day[a]	6,000 ppm in mg/kg/day[a]	10,000 ppm in mg/kg/day[a]
Premating (*P*-gen) - Male	10.5	27.8	320.0	537.9
Premating (*P*-gen) - Female	12.8	31.4	384.1	645.7
Gestation (*P*-gen) - Female	12.9	31.9	393.9	NA[b]
Lactation (*P*-gen) - Female	26.7	74.4	831.0	NA[b]

[a] Individual values were based on the means for each particular phase for each generation.
[b] No pregnancies occurred in the 10,000 ppm dose group.

The concentration of BYI 08330 in the various test diets was analytically verified for batches intended for weeks 1 and 2, 3 and 4, 5 and 6, 9 and 10, 14, 16, and 20 (Bayer Cropscience LP, Environmental Research, Plant and Animal Residue Group, [redacted], [redacted], KS). The homogeneity and stability of BYI 08330 when mixed in the rodent feed was characterized prior to study initiation. The liquid chromatographic methodology used to perform the dietary analyses (i.e., homogeneity, stability, and concentration verifications) required for this study has been described.

Results:

Homogeneity Analysis: The mean concentrations of BYI 08330 in the feed, sampled from three distinct layers in the mixing bowl and containing a nominal concentration of either 70, 150, or 10,000 ppm, were determined to be 68.7 ppm (range 65.0-73.1 ppm; CV = 3.2%), 144 ppm (range 127-153 ppm; CV = 5.5%), and 9,445 ppm (range 8,817-9,943; CV = 3.2%), respectively. Based on a %RSD ≤ to 10%, BYI 08330 was judged to be homogeneously distributed in the feed over a concentration range of 70-10,000 ppm.

Stability Analysis: Following 7 days of room temperature storage, the analytically-determined concentration of the AI of the test substance in the 70-, 150-, or 10,000-ppm admixture was determined to be 68.5 ppm (63.3 ppm on day 0); 131 ppm (151 ppm on Day 0); and 9,564 ppm (9,405 ppm on day 0), respectively. Following 28 days of freezer storage, the analytically-determined concentration of the AI of the test substances in the 70-, 150-, and 10,000-ppm admixtures was determined to be 71.4 ppm (63.3 ppm on day 0); 146 ppm (151 ppm on day 0); and 9,544 ppm (9,405 ppm on day 0), respectively. BYI 08330 mixed in rodent ration was judged to be stable at room temperature for at least seven days and following freezer storage for a minimum of 28 days, over a concentration range of 70-10,000 ppm.

Concentration Analysis: Mean analytical concentrations for each dose group were 197, 498, 5,997, and 10,051 ppm, ranging from 98-101% of the corresponding nominal concentrations of 200, 500, 6,000 and 10,000 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 101% and ranged from 97-103% for rodent ration spiked with 200, 500, 6,000, or 10,000 ppm BYI 08330.

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C. Observations

1. Parental animals:

Mortality and clinical observations. Mortality checks (cageside observations) were performed twice daily (AM and PM), during the workweek and once daily on weekends and holidays with exceptions stated in the Protocol Amendments and Deviations section. Cageside 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 cageside evaluation, the animal was removed from the cage and a detailed assessment conducted. A detailed evaluation of clinical signs includes both observing the animal in the cage and removing the animal to perform a physical examination and was conducted once per week throughout the entire in-life phase of the study.

Body weights and food consumption: Body weights and food consumption were measured and fresh feed provided once/week for both males and females during the 10-week premating period. During the mating period and until sacrifice, body weights for the males were taken once per week. Also during the mating period, fresh feed was provided for both males and unmated females once/week but food consumption was not measured. During gestation, dam body weights were measured on Days 0, 6, 13, and 20, and fresh feed was provided and food consumption measured once/week. During lactation, dam body weights were measured on Days 0, 4, 7, 14, and 21. Fresh feed was provided and food consumption measured once/week, with the exception of week one when food consumption was measured twice (Days 0-4 and 4-7).

Estrous cyclicity:. Estrous cycling was not performed in this one-generation study.

Sperm parameters:. For all 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). Morphology and counts were conducted for all groups.

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2. Litter observations: According to the report, the following litter observations (X) were made.

Observation	Time of observation (lactation day)						
	Day 0	**Day 4** [a]	**Day 4** [b]	**Day 7**	**Day 14**	**Day 21**	**Days (0-21)**
Number of live pups							X
Pup weight	X	X		X	X	X	
External alterations	X	X		X	X	X	
Number of dead pups							X
Sex of each pup (M/F)	X						
Preputial Separation	Performed postweaning						
Vaginal Patency	Performed postweaning						

a Before standardization (culling)
b After standardization (culling)

The size of each litter was adjusted on lactation Day 4 to yield, as close 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 intracranial injection of 0.01-0.05cc Fatal Plus ( ), 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 (lactation day 21). At 21-days of age, a sufficient number of F_1 pups/sex/litter were maintained and observed for vaginal opening and preputial separation. The F_1-males were maintained for an additional 5 to 6 weeks prior to performing sperm analysis (motility, counts, and morphology). F_1-pups not selected for further evaluations were sacrificed and examined macroscopically for any structural abnormalities or pathological changes, particularly as they may relate to the organs of the reproductive system.

Dead pups were examined grossly for external and internal abnormalities, and a possible cause of death was determined for pups stillborn or found dead.

3. Postmortem observations:

1) Parental animals: All surviving parental males were sacrificed as soon as possible after the last litters were produced. Maternal animals were sacrificed following the weaning of their respective litters (lactation day 21). These animals were subjected to postmortem examinations as follows.

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Male rats were sacrificed by carbon dioxide asphyxiation, terminal body weights were taken, and a gross external examination was performed. For all males at termination, sperm was collected from one testes 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).

Each dam was terminated by carbon dioxide asphyxiation and a gross external examination was performed. Terminal body weights were taken and the abdomen and thoracic cavities were opened, and a gross internal examination was performed.
The uterus was excised and the implantation sites, if present, were counted.

Females which were sperm positive and/or had an internal vaginal plug but did not deliver were sacrificed after gestation day 24. Females which 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 deionized water.

The following tissues were collected and weighed (XX). Micropathology (in addition to collection and weights) was performed on those tissues designated with (XXX):

XX	Ovaries	XXX	Testes
XX	Uterus	XXX	Epididymides
XX	Brain	XX	Prostate
XX	Pituitary	XX	Seminal vesicles
XX	Liver	XX	Kidney
XX	Spleen	XX	Thyroid
XX	Thymus	XX	Adrenal

Animals found moribund while on study were sacrificed if their status dictated and a gross necropsy was performed. Animals found dead were necropsied as soon as possible. Necropsy examinations included those parameters previously described.

2) Offspring: Approximately half of the F_1 offspring were sacrificed at 21 days of age. These animals were subjected to postmortem examinations (macroscopic and/or microscopic examination) as follows. The following tissues from 21-day weanlings were collected and weighed: Brain, thymus, spleen, and uterus. Any gross lesion was documented and collected. Exceptions are stated in the Protocol Amendments and Deviations section.

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At 21-days of age, a sufficient number of F_1 pups/sex/litter were maintained for an additional 5 to 6 weeks prior to performing sperm analysis (motility, counts, and morphology).

Pups found dead or terminated in moribund condition underwent a gross necropsy for possible defects and/or cause of death.

D. Data analysis

1. Statistical analyses: The data was analyzed using applications provided by DATATOX (Instem Computer Systems), SAS (SAS Institute, Inc.), or TASC (Toxicology Analysis Systems Customized). Parametric data (including body weight gain and food consumption) was 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) was 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) was 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 was first examined visually, then, in the event of questionable distribution, by statistical analysis using the Chi-square and Fisher's exact tests. Differences between the control and test compound-treated groups was considered statistically significant when $p \leq 0.05$ or $p \leq 0.01$.

2. Indices:

Reproductive indices: The following reproductive indices were calculated from breeding and parturition records of animals in the study:

$$\text{Mating Index (\%)} = \frac{\text{No. inseminated females}^{a}}{\text{No. of females co-housed}} \times 100$$

$$\text{Fertility Index (\%)} = \frac{\text{No. of pregnant females}^{b}}{\text{No. of inseminated females}} \times 100$$

$$\text{Gestation Index (\%)} = \frac{\text{No. of females with live pups}}{\text{No. of pregnant females}} \times 100$$

[a] Includes pregnant females not observed sperm positive or with an internal vaginal plug.
[b] Includes females which did not deliver, but had implantation sites.

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Offspring viability indices: The following viability indices were calculated from lactation records of litters in the study:

$$\text{Birth Index (\%)} = \frac{\text{total No. of pups born/litter}}{\text{total No. of implantation sites/litter}} \times 100$$

$$\text{Livebirth Index (\%)} = \frac{\text{No. of live pups born/litter}}{\text{total No. of pups/litter}} \times 100$$

$$\text{Viability Index (\%)} = \frac{\text{No. of live pups/litter on day 4 (pre-culling)}}{\text{No. of live pups born/litter}} \times 100$$

$$\text{Lactation Index (\%)} = \frac{\text{No. of live pups/litter on day 21}}{\text{No. of live pups/litter on day 4 (post-culling)}} \times 100$$

3. Historical control data: Historical control data are provided in Attachment IV of the original report. This data was obtained from reproduction studies performed in this laboratory (1998 -2004) in the Wistar rat.

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II. Results

Findings:

a) Parental toxicity

General observations: There were no treatment-related mortalities or clinical signs observed during the course of the study. Females showed nonstatistical body weight gain declines at 6,000 ppm during during lactation (12%). Females of the 10,000 ppm group were sacrificed after the gestation phase due to non-pregnancy (see below). Food consumption was not affected in either the males or females at any dietary level tested.

Table 5.6.1-1: Mean (s.d.) body weight (g) and body weight gain (g)

Body weight	Dose Group (ppm)				
	0	200	500	6,000	10,000 [a]
P generation males (pre-mating)					
BW – week 0	**325.4**	**329.3**	**325.6**	**325.8**	**326.7**
BW – week 10	**441.3**	**444.4**	**439.4**	**438.3**	**431.4**
BW gain – week 1-10	**115.9**	**115.1**	**113.8**	**112.5**	**104.7**
P generation females (pre-mating)					
BW – week 0	**202.1**	**199.6**	**202.8**	**200.3**	**202.1**
BW – week 10	**246.1**	**245.1**	**247.2**	**239.4**	**240.7**
BW gain – week 1-10	**44.0**	**45.5**	**44.4**	**39.1**	**38.6**
P generation females (lactation)					
BW LD 0	**273.2**	**270.6**	**262.1**	**263.6**	
BW LD 4	**282.2**	**280.4**	**276.1**	**273.3**	
BW LD 7	**289.4**	**288.6**	**282.8**	**279.8**	
BW LD 14	**306.8**	**304.6**	**301.5**	**291.5**	
BW LD 21	**293.4**	**290.1**	**286.3**	**281.4**	

a = no pups at this dietary level; * = $p < 0.05$; s.d. = standard deviation

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b) Reproductive function: In P generation males a significant decline in both motility and progression of sperm was noted in the 10,000 ppm dose group as well as a slight decline in epididymal counts and a significant increase in abnormal sperm. The abnormal sperm observed in the morphological exam presented as amorphous sperm heads. These findings resulted in no pregnancies at this dietary level. Sperm analysis revealed no effects in P generation males at 6,000 ppm and below.

In F1 eight-to-nine week old interim males, slight declines in motility and progression of sperm was observed in the 6,000 ppm dose group. Morphology showed an increase in abnormal sperm presenting as amorphous sperm heads. There were no sperm effects observed in F1 eight-to-nine week old interim animals at 500 ppm and below.

Table 5.6.1-2: Mean sperm measures

Sperm Analysis	Dose Group (ppm)				
	0	200	500	6,000	10,000 [a]
P generation males (n≈10/group)					
Motility: % motile	**74.1**	**74.0**	**79.0**	**75.2**	**30.6**
Motility: % progressive	**57.4**	**53.7**	**58.3**	**52.4**	**14.1**
Sperm counts: sperm/g testis	**98.8**	**128.4**	**106.7**	**111.0**	**97.7**
Sperm counts: sperm/g epididymis	**985.6**	**732.8**	**798.1**	**944.2**	**464.0**
Morphology: number of normal cells	**199.2**	**199.3**	**199.5**	**199.0**	**105.0**
Morphol.: number of amorphous heads	**0.7**	**0.7**	**0.5**	**0.8**	**95.5**
Morphology: number of small heads	**0.1**	**0.0**	**0.0**	**0.2**	**0.0**
F1 eight-to-nine week old interim animals (n≈15/group)					
Motility: % motile	**89.5**	**89.7**	**88.3**	**75.5**	
Motility: % progressive	**69.2**	**63.5**	**62.5**	**55.7**	
Sperm counts: sperm/g testis	**81.8**	**75.1**	**73.1**	**73.0**	
Sperm counts: sperm/g epididymis	**503.6**	**485.7**	**507.1**	**463.4**	
Morphol.: number of normal cells	**197.2**	**198.5**	**198.7**	**179.6**	
Morphol.: number of amorphous heads	**2.8**	**1.5**	**1.3**	**20.2**	
Morphol.: number of small heads	**0.1**	**0.0**	**0.0**	**0.0**	

a = no pups at this dietary level

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c) Reproductive performance: The 10,000 ppm dietary level was associated with no fertility in the P generation animals. There were no implantation sites noted in the females of this same level. At the 200-, 500-, and 6,000-ppm dietary levels, there were no compound-related effects on any parameter (e.g., mating, fertility, or gestation indices, days to insemination, gestation length, or the median number of implants). The litter parameters determined at birth (litter size, percentage of males born, birth index, live birth index, viability index, lactation index), were not affected.

Gross pathology, organ weights: No treatment-related macroscopic changes were observed at necropsy in P generation rats. Absolute and relative weight of the cauda epididymis was decreased in the 10,000 ppm dose group. In the females of the 10,000 ppm dose group, terminal body weight declines, as well as, declines in absolute and relative kidney and liver weights were observed. However, a direct compound-related effect cannot be determined due to the comparison of nonpregnant females (10,000 ppm group) with pregnant controls (sacrificed on lactation day 21).

Table 5.6.1-3: Affected organ weight (g)

Weight (g) ± s.d.	Dose Group (ppm)				
	0	200	500	6,000	10,000
P generation males					
Cauda epid. abs.	**0.337±0.038**	**0.321±0.022**	**0.346±0.036**	**0.311±0.071**	**0.239±0.030** *
Cauda epid. rel.	**0.074±0.009**	**0.071±0.005**	**0.076±0.009**	**0.069±0.017**	**0.054±0.005** *
P generation females					
Body weight	**283.4 ± 26.0**	**279.1 ± 24.7**	**280.4 ± 25.2**	**272.8 ± 22.7**	**254.5 ± 16.2**
Liver, abs.	**11.318**	**11.544**	**12.131**	**12.039**	**8.531** *
Liver, rel.	**3.963**	**4.103**	**4.313**	**4.394**	**3.351**
Kidney, abs.	**2.092**	**2.044**	**2.044**	**1.956**	**1.732** *
Kidney, rel.	**0.738**	**0.731**	**0.729**	**0.717**	**0.681**

s.d. = standard deviation; epid. = epididymis; abs. = absolute; rel. = relative; * = $p < 0.05$

Histopathology: P generation males in the 10,000 ppm group showed abnormal sperm in the epididymis (10/10) and cauda epididymis (9/9). The abnormal sperm consisted of what appears to be the retention of the residual body to the tail of the spermatozoa. Severity grade consisted of minimal to moderate change. There was no evidence of change in the epithelium lining the seminiferous tubules of the testes in 10,000 ppm generation males. There were no abnormal sperm cells observed in P generation males at 6,000 ppm and below.

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d) Neonatal toxicity

General observations: No treatment-related clinical signs and no malformations were observed in F1 pups. Pup weights at birth were not affected. At 6,000 ppm, significant declines in offspring body weight (13.9%) were noted at lactation day 21 with a total decline in body weight gain of 14.7%.

Table 5.6.1-4: Mean (s.d.) Pup Body Weights (g) –male and female-

Lactation Day	Dose Group (ppm)									
	0		200		500		6,000		10,000 [c]	
F1 pups (21 day old)										
0	**6.3 ± 0.25**		**6.1 ± 0.21**		**5.8 ± 0.09**		**5.9 ± 0.19**			
4 [a]	**10.9 ± 0.59**		**10.5 ± 0.68**		**9.9 ± 0.1**		**10.1 ± 0.40**			
4 [b]	**10.9 ± 0.57**		**10.5 ± 0.69**		**9.9 ± 0.1**		**10.0 ± 0.41**			
7	**17.4 ± 0.87**		**16.7 ± 1.01**		**15.9 ± 0.24**		**15.8 ± 0.53**			
14	**34.9 ± 1.84**		**33.4 ± 1.21**		**31.9 ± 0.68**		**31.5 ± 0.80**			
21	**53.1 ± 2.38**		**50.1 ± 1.57**		**48.4 ± 0.89**		**45.7 ± 1.17**	*		
Gain	**46.8**		**44.0**		**42.6**		**39.9**	*		

a= before standardization (culling); b=after standardization; c = no pups at this dietary level,
* = $p < 0.05$; s.d. = standard deviation

Gross pathology, organ weights: In F1 pups of 21 days of age no treatment-related macroscopical alterations were observed. For the pups selected for microscopic examinations, no significant differences in terminal body weights or absolute and relative organ weights were noted at any dietary level tested.

In F1 eight-to-nine week old interim males significant declines in terminal body weight was observed at 6,000 ppm. There were no statistically significant effects observed on the organs weighed (testis, epididymis, cauda epididymis) at any dietary level tested.

Table 5.6.1-5: Mean (s.d.) terminal body weights (g)

Body weight	Dose Group (ppm)									
	0		200		500		6,000		10,000 [a]	
F1 eight-to-nine week old interim animals										
Terminal BW	**278.4**		**289.6**		**277.6**		**257.0**	*		

a = no pups at this dietary level; * = $p < 0.05$; s.d. = standard deviation

Histopathology: In F1 weanlings the testis and epididymis of the control and 6,000 ppm animals did not contain sperm due to the immaturity of the animals. The epithelium appeared normal.

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F1 eight-to-nine week old interim animals of the 6,000 ppm dose group showed abnormal sperm in the epididymis (4/15) and cauda epididymis (4/14). Severity grade consisted of minimal or moderate change. A single control animal had a moderate number of abnormal sperm within the epididymis and cauda epididymis. There was no evidence of change in the epithelium lining the seminiferous tubules of the testes in F1 eight-to-nine week old interim animals in the 6,000 ppm group. There were no abnormal sperm cells observed in F1 eight-to-nine week old interim animals at 500 ppm and below.

Sexual maturation: There were no treatment-related findings observed in the F1 generation on either preputial separation or vaginal patency at any dietary level tested.

Conclusion:

The NOAEL for male parental toxicity in this pilot study was established at 6,000 ppm (equal to premating doses of 320 mg/kg bw/day), based on reduced cauda epididymal weights and effects on sperm at 10,000 ppm (equal to 538 mg/kg bw/day). The highest dietary exposure level (10,000 ppm) caused treatment-related effects on sperm cells of P generation males and included decreased sperm motility and progression, decline in epididymal sperm count and increase in abnormal sperm in the epididymis and the cauda epididymis.

The NOAEL for female parental toxicity in this pilot was established at 500 ppm (equal to lactating doses of 74.4 mg/kg bw/day), based on slight body weight gain declines during lactation in the P-generation females at 6000 ppm (equal to 831 mg/kg bw/day).

The NOAEL for male reproductive toxicity in this pilot study was established at 6,000 ppm in P-generation males (equal to premating doses of 320 mg/kg bw/day), based on decreased sperm motility and progression, decreased epididymal counts, increase in abnormal sperm, in the epididymis and the cauda epididymis in P-generation males at 10,000 ppm (equal to 538 mg/kg bw/day).

Other than the lack of pregnancies in the 10,000 ppm dose group, considered to be attributed to the effects on the sperm of the males, there were no reproductive effects observed in the females during the course of this pilot study.

The NOAEL for neonatal toxicity in this pilot study was established at 500 ppm, based on decreased body weights on lactation day 21 at 6,000 ppm. Pup weight declines corresponded with maternal body weight declines.

The NOAEL for male parental toxicity in F1 eight-to-nine week old interim males in this pilot study was established at 500 ppm based on decreased terminal body weights at 6,000 ppm.

The NOAEL for male reproductive toxicity in F1 eight-to-nine week old interim males in this pilot study was established at 500 ppm based on decreased sperm motility and progression, increase in abnormal sperm in the epididymis and the cauda epididymis at 6,000 ppm most likely caused by a higher dietary intake of BYI 08330 and subsequent higher exposure to the test material when compared to the corresponding non-affected P generation males at 6,000 ppm which were exposed to average daily doses of 320 mg/kg bw/day. This is due to the fact that F1 interim males were younger at the start of dietary treatment when compared to P generation males and necessarily consumed more feed and chemical substance in relation to their body weight.

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Report:	**KIIA 5.6.1/02, ; 2006**
Title:	Technical grade BYI 08330: A Two Generation Reproductive Toxicity Study in the Wistar Rat
Report No & Document No	201426-1 M-274619-02-1
Guidelines:	OECD 416, OPPTS 870.3800, MAFF 12-Nousan-No. 8147
OECD/FIFRA GLPS	Yes (certified laboratory). Deviations: none

Executive Summary

In the present two-generation study, groups of 30 male and 30 female Wistar Hanover rats (strain: Crl:WI(Glx/BRL/Han)IGS BR) were exposed to BYI 08330, batch no. 08045/0014, purity: 97.4 – 98.5%, at dietary concentrations of 0, 250, 1,000 or 6,000 ppm. The dietary concentrations were equal to premating doses of 0, 17.2, 70.7 and 419.3 mg/kg bw/day in P-generation males, to 0, 19.3, 79.5 and 486.7 mg/kg bw/day in F1-generation males, to 0, 20.0, 82.5 and 484.7 mg/kg bw/day in P-generation females, and to 0, 21.7, 90.3 and 539.5 mg/kg bw/day in F1-generation females, respectively. P-generation rats were approximately 8-9 weeks of age at the time of study initiation, corresponding to body weights of 240.9 - 307.7 g. They were pretreated for ten weeks, during the subsequent maximum one-week mating period and until the last litters were produced (males) or until lactation day 21 (females). F1 offsprings were nursed up to an age of three weeks. Some of them were selected for further treatment and for breeding a F2 generation at 5-9 weeks of age.

The study revealed parental toxicity at 6,000 ppm. Effects included body weight gain declines in males of either generation, and decreased terminal body weights, increased multifocal renal tubular dilatation in F1-generation males. In females of both generations, reduced food consumption was noted at 6,000 ppm during lactation. In addition, F1-generation females of the 6,000 ppm group showed declines in body weights and in body weight gain at the end of the premating phase, decreased terminal body weights and increased multifocal renal tubular dilatation.

Overall reproductive performance revealed no effects on any parameter measured in either generation at 6,000 ppm and below. The litter parameters determined at birth were not affected in both generations. Histopathology of the reproductive tract of females of either generation showed no treatment-related findings. Ovarian follicle counts of 6,000-ppm dose F1-generation females showed no difference to controls. Female reproductive function (estrus cycle staging) was not affected in both P- and F1-generation at any dietary level tested.

In males, reproductive function (sperm analysis: sperm count, motility, progression, and morphology as well as gross examination, weight and histopathology of reproductive organs) was not affected in the P-generation at 6,000 ppm (=419.3 mg/kg bw/day) and below.
In F1-generation males, sperm motility, epididymal and testicular sperm count was also not affected at 6,000 ppm. However, abnormal sperm cells were still noted at 6,000 ppm (=486.7 mg/kg bw/day), with a high degree of variation as 9/30 males exhibited a minimal effect (1 to 4 abnormal sperms was noted out of 200 cells viewed) but only one outlying male was affected to the extent that it compromised its fertilizing capabilities (i.e. 82% total abnormal sperm with 77% of these sperm

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presenting as amorphous heads). In histopathology, only this same male showed abnormal sperm in the epididymis. Reproductive function was not affected in F1-generation males at 1,000 and 250 ppm.

Neonatal toxicity was limited to decreased body weights in F1 litters on lactation day 21 (8.2%) and in F2 litters on lactation day 14 (7.5%) and lactation day 21 (10.3%). Pup weights at birth were not affected. Pup weight declines corresponded with maternal body weight and food consumption declines. There were no treatment-related effects on the viability parameters (viability index, lactation index), and no clinical signs and no malformations were observed in both F1 and F2 pups. Gross necropsy, organ weights (brain, thymus, spleen, uterus) and histopathology of the reproductive organ system revealed no direct compound-related findings at any dietary levels tested. Maturation of external sexual organs (balano-preputial separation, vaginal opening) was not affected in F1 weanlings.

The NOAEL for male parental toxicity was established at 1,000 ppm (equal to premating doses of 70.7 mg/kg bw/day or 79.5 mg/kg bw/day in P- or F1-generation males, respectively), based on body weight gain declines in P-generation males at 6,000 ppm (equal to 419.3 mg/kg bw/day) and body weight gain declines, decreased terminal body weights and increased renal multifocal tubular dilatation in F1-generation males at 6,000 ppm (equal to 486.7 mg/kg bw/day). Marginal effects on reproductive function (abnormal sperm) occurred in the F1-generation males at the high dose level of 6,000 ppm, a dose level which elicits also parental toxicity; P-generation males were not affected. Reproductive performance was not affected at 6,000 ppm in either generation.
The NOAEL for female parental toxicity was established at 1,000 ppm (equal to premating doses of 82.5 mg/kg bw/day or 90.3 mg/kg bw/day in P- or F1-generation females, respectively), based on reduced food consumption during lactation in P-generation females at 6,000 ppm(equal to 484.7 mg/kg bw/day) and reduced food consumption during lactation, reduced body weights (end premating), body weight gain declines (premating), decreased terminal body weights and increased renal multifocal tubular dilatation in F1-generation females at 6,000 ppm (equal to 539.5 mg/kg bw/day).

The NOAEL for male reproductive toxicity is 1,000 ppm (equal to premating doses of 79.5 mg/kg bw/day of F1 males), based on abnormal sperm cells at 6,000 ppm (equal to 486.7 mg/kg bw/day). The difference in outcome between 6,000 ppm P-generation males (no sperm effects) and 6,000 ppm F1-generation males (slight sperm effects) is most likely due to the higher dietary intake and subsequent higher exposure to the test material in F1-generation males (486.7 mg/kg bw/day) when compared to P-generation males (419.3 mg/kg bw/day).
The NOAEL for female reproductive toxicity was established at 6,000 ppm (equal to premating doses of 484.7 mg/kg bw/day or 539.5 mg/kg bw/day in P- or F1-generation females, respectively) based on the absence of effects at 6,000 ppm.

The NOAEL for neonatal toxicity is 1,000 ppm, which is the same as for parental toxicity. It is based on slight growth retardation at 6,000 ppm corresponding with declines in maternal food consumption and body weight. Developmental landmarks were not affected by treatment with BYI 08330.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	White Powder
Lot/Batch No.:	08045/0014
Purity:	97.8% (12/03), 97.6% (6/04), 98.5% (1/05), 97.4% (5/05)
Compound Stability:	stable at room temperature (~22°)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	Non-stereo: 382608-10-8; cis: 203313-25-1

2. Vehicle and/or positive control: None.
Compound was dry mixed and administered through the diet.

3. Test animals:

Species:	Rat, males and females
Strain:	Wistar Hanover rats Crl:WI(Glx/BRL/Han)IGS BR
Number of animals:	Total animals ordered: 280; Number used on study: 130 male and 130 female 10 animals/sex were used for vendor surveillance, which included a gross necropsy and serological evaluation. 5 animals/sex were sacrificed prior to initiation of exposure and 5 animals/sex were sacrificed approximately four weeks after initiation of exposure.
Age at study initiation:	(P) 8-9 wks
Wt. at study initiation:	(P) Males: 240.9 - 307.7 g; Females: 175.4 - 213.5 g
Source:	[redacted]
Animal Receipt:	Upon receipt, animals were examined by a veterinarian prior to release for study.
Housing:	Animals passing the initial shipment exam were housed individually (except during the mating phase and as noted below for the F_1 pups) in suspended stainless steel cages and deotized cage board in the bedding trays. During gestation and lactation, individual dams (and litters) were housed in polycarbonate cages with corn-cob bedding
Diet:	*ad libitum* - [redacted] Chow 5002 meal
Water:	*ad libitum* - pressure-activated water nipples

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Environmental conditions:	**Temperature:**	Room temperature 18 to 26ºC
	Humidity:	Relative humidity 30 to 70%, exceptions stated in the Protocol Amendments and Deviations section
	Air changes:	Minimum of 10 air changes per hour, based on average number of changes per hour per day
	Photoperiod:	Daily photoperiod of 12 hrs of light [6:00 a.m. to 6:00 p.m.] alternating with 12 hrs of darkness, exceptions stated in the Protocol Amendments and Deviations section
Acclimatisation:	8 days	

All procedures associated with the animals were approved by the Institution's and Use Committee (IACUC); and during the course of the study, room conditions were monitored and recorded in a consistent and timely manner. Feed and water samples were collected periodically and analyzed for potential contaminants; the concentrations outlined in the "Certification Profile" for Chows were used as a standard of comparison for judging the acceptability of any contaminants detected in the analyzed feed samples.

B. Procedures and study design

1. Mating procedure: Males and females were exposed to the test material for ten weeks prior to mating. Mating was accomplished by co-housing one female with one male for up to 14 consecutive days with exceptions stated in the Protocol Amendments and Deviations section. Approximately four to six animals from each dose group were co-housed daily beginning on the first day of the mating phase and continuing until all animals had been cohoused. 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 which may 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.

2. Study schedule: One hundred and twenty female and one hundred and twenty male rats were assigned to one of four treatment groups (30 animals/group): nominal doses of 0, 250, 1,000, and 6,000 ppm technical grade BYI 08330 in the diet. Animals were exposed to the treated feed throughout the entire study. The parental animals (*P* and F_1-generations) were given test diets for 10 weeks prior to mating. Selection of F_1-pups to evaluate preputial separation and vaginal patency was made when the pups were 21-days of age. The F_2-generation pups were maintained until 21-days old.

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3. Animal assignment: Following a minimum of 6 days of acclimation and prior to initiation of treatment, study animals were examined by a veterinarian and released for study use. The animals were then assigned to either a control or one of three chemically-treated groups using a weight stratification-based computer program (INSTEM Computer Systems, Stone, Staffordshire, UK) and those animals falling within +/- 20% of the mean for all animals were put on study. Once animals were distributed into their dose groups, each rat on study had a microchip (, Delaware) subcutaneously implanted on their dorsal surface in the region between their scapulae. At the very least, 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. All animals not placed on study (for whatever reason) were ultimately sacrificed; however, prior to that time the animals were available, subject to the approval of the IACUC, for alternative uses which may have been unrelated to this study.

Test Group	Dose in Diet [a] (ppm)	Animals/group			
		P Males	P Females	F_1 Males	F_1 Females
Control	0 ppm	30	30	30	30
Low (LDT)	250 ppm	30	30	30	30
Mid (MDT)	1,000 ppm	30	30	30	30
High (HDT)	6,000 ppm	30	30	30	30

a Diets were administered from beginning of the study until sacrifice

4. Dose selection rationale: Doses were selected based upon the preliminary results which emerged in the rat over the course of a pilot reproductive toxicity testing study conducted with the test chemical at doses of 0, 200, 500, 6,000 and 10,000 ppm BYI 08330/kg body weight/day (Report No. 201300-1). In that study, the *P*-generation produced no births in the 10,000 ppm dose group in the absence of effects on body weight, food consumption or clinical observations. In this same dose group, declined epididymal weights were observed. Sperm analysis revealed declined sperm motility (67%) and % progressive sperm (79%), as well as, abnormal sperm in the epididymis and cauda epididymis. The F_1-generation 8-9 week old males of the 6,000 ppm dose group also exhibited, to a much lesser extent, declined sperm motility (15%) and % progressive sperm (15%), as well as, abnormal sperm in the epididymis and cauda epididymis. Based on these interim results, the doses selected for the two-generation reproduction toxicity study were 0, 250, 1,000 and 6,000 ppm BYI 08330.

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5. Dosage preparation and analysis: The test compound was mixed directly 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 taken directly from the bag without mixing. 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, for each week, were stored under freezer conditions until presented to the animals the following week. Additionally, the entire batch of replacement admixture for each treatment group was used for subsequent weekly feedings if within freezer stability limits.

Feed samples were taken from each dietary level and analyzed for concentration for study weeks 1, 2, 3 and at monthly intervals thereafter.

Phase of Study	250 ppm in mg/kg/day[a]	1,000 ppm in mg/kg/day[a]	6,000 ppm in mg/kg/day[a]
Premating (P-gen) - Male	17.2	70.7	419.3
Premating (P-gen) - Female	20.0	82.5	484.7
Gestation (P-gen) - Female	19.6	76.7	467.4
Lactation (P-gen) - Female	39.4	162.9	895.7
Premating (F_1 -gen) - Male	19.3	79.5	486.7
Premating (F_1 -gen) - Female	21.7	90.3	539.5
Gestation (F_1 -gen) - Female	17.8	69.8	434.7
Lactation (F_1 -gen) - Female	39.4	161.0	930.6

[a] Individual values were based on the means for each particular phase for each generation.

Results

Homogeneity Analysis: The homogeneity and stability of the test substance in rodent feed was verified. The mean concentrations of BYI 08330 in the feed, sampled from three distinct layers in the mixing bowl and containing a nominal concentration of either 70, 150, or 10,000 ppm, were determined to be 68.7 ppm (range 65.0-73.1 ppm; CV = 3.2%), 144 ppm (range 127-153 ppm; CV = 5.5%), and 9,445 ppm (range 8,817-9,943; CV = 3.2%), respectively.
Based on a %RSD ≤ to 10%, BYI 08330 was judged to be homogeneously distributed in the feed over a concentration range of 70-10,000 ppm.

Stability Analysis: Following 7 days of room temperature storage, the analytically-determined concentration of the AI of the test substance in the 70-, 150-, or 10,000-ppm admixture was determined to be 68.5 ppm (63.3 ppm on day 0); 131 ppm (151 ppm on day 0); and 9,564 ppm (9,405 ppm on day 0), respectively. Following 28 days of freezer storage, the analytically-determined concentration of the AI of the test substances in the 70-, 150-, and 10,000-ppm admixtures was determined to be 71.4 ppm (63.3 ppm on day 0); 146 ppm (151 ppm on day 0); and 9,544 ppm (9,405 ppm on day 0), respectively.

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BYI 08330 mixed in rodent ration was judged to be stable at room temperature for at least seven days and following freezer storage for a minimum of 28 days, over a concentration range of 70-10,000 ppm.

Concentration Analysis: The concentration of BYI 08330 in the various test diets was determined using liquid chromatography. Mean analytical concentrations for each dose group were 244, 1,006 and 5,898 ppm, ranging from 98-101% of the corresponding nominal concentrations of 250, 1,000 and 6,000 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 99% and ranged from 94-103% for rodent ration spiked with 250, 1,000 or 6,000 ppm BYI 08330.

C. Observations

1. Parental animals:

Mortality and clinical observations: Mortality checks (cageside observations) were performed twice daily (AM and PM), during the workweek and once daily on weekends and holidays. Cageside 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 cageside evaluation, the animal was removed from the cage and a detailed assessment conducted. A detailed evaluation of clinical signs, which included both observing the animal in the cage and removing the animal to perform a physical examination, was conducted once per week throughout the entire in-life phase of the study.

Body weights and food consumption: Body weights and food consumption were measured and fresh feed provided once/week for both males and females during the 10-week premating period with exceptions stated in the Protocol Amendments and Deviations section. During the mating period and until sacrifice, body weights for the males were taken once per week. Body weights were also taken during the mating period for unmated females. Also during the mating period, fresh feed was provided for both males and unmated females once/week but food consumption was not measured. During gestation, dam body weights were measured on days 0, 6, 13 and 20, and fresh feed was provided and food consumption measured once/week. During lactation, dam body weights were measured on days 0, 4, 7, 14 and 21. Fresh feed was provided and food consumption measured once/week, with the exception of week one 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.

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Sperm parameters: For all *P* and F_1-generation males at termination, sperm was collected from one testes 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 the IVOS. Morphology and testis counts were conducted on the control and highest dose group and in all dose groups for epididymal counts for the first generation. Morphology and counts were conducted on all dose groups for the second generation.

2. Litter observations: The following litter observations (X) were made.

Observation	Time of observation (lactation day)						
	Days (0-21)	Day 0	Day 4 [a]	Day 4 [b]	Day 7	Day 14	Day 21
Number of live pups	X						
Pup weight		X	X		X	X	X
External alterations		X	X		X	X	X
Number of dead pups	X						
Sex of each pup (M/F)		X					
Preputial Separation	Performed postweaning for F_1 pups						
Vaginal Patency	Performed postweaning for F_1 pups						

a Before standardization (culling)
b After standardization (culling)
Exceptions stated in the Protocol Amendments and Deviations section

The size of each litter was adjusted on lactation Day 4 to yield, as close as possible, four males and females per litter. If the number of male or female pups was less than four, a partial adjustment was made (e.g., three males and five females). 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 intracranial injection of 0.01-0.05cc Fatal Plus ( ). 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 (lactation day 21). At 21-days of age a sufficient number of F_1-pups/sex/litter were maintained to produce the next generation. F_1-pups not selected to become parents of the next generation and all F_2-pups were sacrificed and examined macroscopically for any structural abnormalities or pathological changes, particularly as they may relate to the organs of the reproductive system.
Dead pups were examined grossly for external and internal abnormalities and a possible cause of death was determined for pups stillborn or found dead.

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3. Postmortem observations:

1) Parental animals: All surviving parental males were sacrificed as soon as possible after the last litters were produced. Maternal animals were sacrificed following the weaning of their respective litters (lactation day 21). These animals were subject to examinations as follows.

P-generation males and females (10 animals/sex/level) were bled from the orbital sinus utilizing a glass capillary tube under brief isofluorane anesthesia for possible evaluation of clinical chemistry parameters. Liver samples were also collected for the same animals and immediately placed in liquid nitrogen prior to placing in -70ºC freezer for possible evaluation of liver enzymes. Analysis of clinical chemistries and liver enzymes was deemed unnecessary.

Male rats were sacrificed by carbon dioxide asphyxiation, terminal body weights were taken, and a gross external examination was performed. For all males at termination, sperm was collected from one testes 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).

Each dam was terminated by carbon dioxide asphyxiation and a gross external examination was performed. Terminal body weights were taken and the abdomen and thoracic cavities were opened and a gross internal examination was performed. The uterus was excised and the implantation sites, if present, were counted.

Females which were found sperm positive and/or had an internal vaginal plug but did not deliver were sacrificed after gestation Day 24. Females which 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 deionized water.

The following tissues were collected (X) and weighed (XX). Micropathology (in addition to collection and weights) was performed on those tissues designated with (XXX):

XX	Brain	XX	Thyroid	XXX	Prostate	X	Cervix
XXX	Pituitary	XX	Thymus	XXX	Seminal Vesicle	X	Vagina
XXX	Liver	XXX	Adrenal	XXX	Testis	X	Oviduct
XXX	Kidney	XXX	Epididymis	XXX	Uterus	X	Coagulating Gland
XXX	Spleen	XXX	Ovary			X	Gross Lesions

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Animals found moribund while on study were sacrificed if their status dictated and a gross necropsy was performed. Animals found dead were necropsied as soon as possible. Necropsy examinations included those parameters previously described.

2) 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 subject to postmortem examinations (macroscopic and/or microscopic examination) as follows. The following tissues from 21-day weanlings were collected and weighed: brain, thymus, spleen, and uterus. Any gross lesion was documented and collected. Micropathology was performed on the following tissues: From 1 female/litter; uterus, ovary, vagina, cervix, oviduct (fallopian tube) and from 1 male/litter; testis, epididymis, prostate, coagulating gland, seminal vesicles. Exceptions stated in the Protocol Amendments and Deviations section.

Pups found dead or terminated in a moribund condition underwent a gross necropsy for possible defects and/or cause of death.

D. Data analysis

1. Statistical analyses: The data was analyzed using applications provided by DATATOX (Instem Computer Systems), SAS (SAS Institute, Inc.), or TASC (Toxicology Analysis Systems Customized). Parametric data (including body weight gain and food consumption) was 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) was first analyzed by the Kruskal-Wallis test and then by the Dunn's Test if significant differences were identified. Nonparametric dichotomous data (e.g. fertility and gestation indices) was 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 was first examined visually, then, in the event of questionable distribution, by statistical analysis using the Chi-square and Fisher's exact tests. Differences between the control and test compound-treated groups was considered statistically significant when $p < 0.05$ or $p < 0.01$.

2. Indices: Reproductive indices: The following reproductive indices were calculated from breeding and parturition records of animals in the study:

Mating Index (%) = No. inseminated femalesa x 100 / No. of females co-housed

Fertility Index (%) = No. of pregnant femalesb x 100 / No. of inseminated females

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$$\text{Gestation Index (\%)} = \frac{\text{No. of females with live pups}}{\text{No. of pregnant females}} \times 100$$

a Includes pregnant females not observed sperm positive or with an internal vaginal plug.

b Includes females which did not deliver, but had implantation sites.

Offspring viability indices: The following viability indices were calculated from lactation records of litters in the study:

$$\text{Birth Index (\%)} = \frac{\text{total No. of pups born/litter}}{\text{total No. of implantation sites/litter}} \times 100$$

$$\text{Livebirth Index (\%)} = \frac{\text{No. of live pups born/litter}}{\text{total No. of pups/litter}} \times 100$$

$$\text{Viability Index (\%)} = \frac{\text{No. of live pups/litter on day 4 (pre-culling)}}{\text{No. of live pups born/litter}} \times 100$$

$$\text{Lactation Index (\%)} = \frac{\text{No. of live pups/litter on day 21}}{\text{No. of live pups/litter on day 4 (post-culling)}} \times 100$$

3. Historical control data: Historical control data are provided in Attachment IV of this report. This data was obtained from reproduction studies performed in this laboratory (1998 -2004) in the Wistar rat.

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II Results

Findings:

a) Parental toxicity

General observations:

There were no treatment-related mortalities or clinical signs observed during the course of the study. Toxicologically significant effects on feed intake were determined at 6,000 ppm in females during lactation. Body weight and/or-body weight gain was reduced at 6,000 ppm in males and females, indicating that a maximum tolerated dose (MTD) was reached at this dietary exposure level. Details are as follows:

Premating: A decline in body weight gain (Wk 1-10) was noted in P-generation males (13.2%) of the 6,000 ppm group. In the F1-generation, the males of the 6,000 ppm group exhibited statistically significant declines in body weight (mean of 6.4%) from days 14-70 and declines in overall body weight gain (8%). In F1-generation females of the 6,000 ppm group, statistically significant declines in body weight (5.7%, week 10 of premating) and declines in overall body weight gain (11.3%) were observed at the end of the premating phase.

Gestation: A decline in body weight gain (7.5%) was noted in F1-generation females of the 6,000 ppm group which was accompanied by statistically significantly lower body weight means (6.5%) from day 0 – 20 of gestation.

Lactation: P-generation females of the 6,000 ppm group showed statistically significant declines in food consumption on both a g/kg/day (11.5 %) and g/animal/day (13.4 %) basis. In the F1-generation, females of the 6,000 ppm group exhibited statistically significant body weight declines (grand mean of all body weight means of lactation days 0 – 21: 6.6 %) throughout lactation, as well as, significantly declined food consumption (lactation days 7-21) on both a g/kg/day (8.6 %) and g/animal/day (13.9 %) basis. Statistically significant declines in food consumption were also observed in F1-generation females of the 1,000 ppm group (lactation days 14–21) and are considered equivocal.

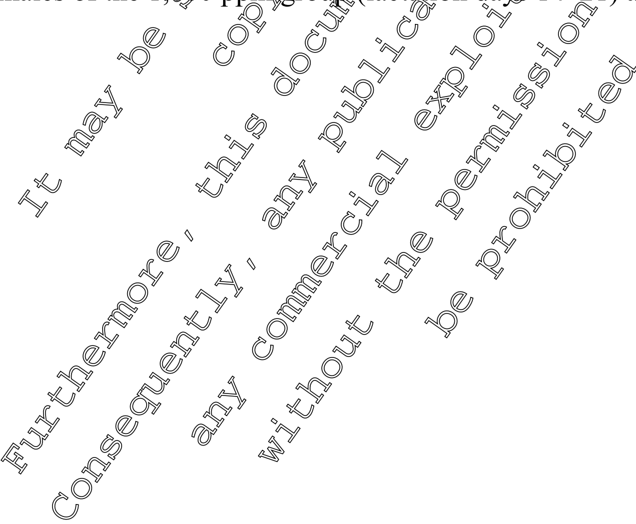

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Table 5.6.1-6: Mean body weights (g)

	Dose Group (ppm)							
	0		250		1,000		6,000	
P generation males (pre-mating)								
Body weight – Wk 0	270.8		273.5		275.6		277.2	
Body weight – Wk 10	430.3		438.5		425.6		415.7	
Weight gain – Wk 1-10	159.5		165.0		150.0		138.5	
F1 generation males (pre-mating)								
Body weight – Wk 0	198.5		198.9		197.1		189.4	
Body weight – Wk 10	401.0		416.0		404.8		375.9	*
Weight gain – Wk 1-10	202.5		217.1		207.7		186.5	
P generation females (pre-mating)								
Body weight – Wk 0	191.7		192.2		194.6		193.2	
Body weight – Wk 10	241.4		244.0		241.7		240.2	
Weight gain – Wk 1-10	49.7		51.8		47.1		47.0	
P generation females (gestation)								
Body weight – GD 20	346.6		344.3		341.9		335.0	
Weight gain – GD 0-20	102.8		98.3		93.9		93.7	
F1 generation females (pre-mating)								
Body weight – Wk 0	147.2		146.2		148.8		143.2	
Body weight – Wk 10	226.7		226.2		226.1		213.7	*
Weight gain – Wk 1-10	79.5		80.0		77.3		70.5	
F1 generation females (gestation)								
Body weight – GD 20	318.0		324.5		310.8		296.7	**
Weight gain – GD 0-20	88.9		95.3		83.8		82.2	
F1 generation females (lactation)								
Body weight – LD 0	247.5		254.7		245.9		232.0	**
Body weight – LD 4	258.9		259.2		255.0		238.6	**
Body weight – LD 7	267.0		268.9		263.1		248.6	**
Body weight – LD 14	277.4		282.8		277.5		260.4	**
Body weight – LD 21	270.2		275.7		264.9		254.4	**

* = p < 0.05, ** = p < 0.01 ; s.d. = standard deviation

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Table 5.6.1-7: Mean food consumption (g/kg bw/day)

	Dose Group (ppm)							
	0		250		1,000		6,000	
P generation females (lactation)								
Food cons. – LD 0-4	124.1		111.4		108.3	*	106.5	**
Food cons. – LD 4-7	164.4		152.4		151.8		144.4	**
Food cons. – LD 7-14	181.6		175.0		179.7		165.5	**
Food cons. – LD 14-21	214.0		207.5		208.0		191.1	**
F1 generation females (lactation)								
Food cons. – LD 0-4	117.7		107.3		104.3		109.0	
Food cons. – LD 4-7	156.9		147.1		153.2		158.2	
Food cons. – LD 7-14	185.4		182.1		184.9		169.8	**
Food cons. – LD 14-21	212.8		208.5		197.8	*	194.1	**

** = p < 0.01

Gross pathology, organ weights: No treatment-related macroscopic changes were observed at necropsy in P- and F1-generation rats.
Significant declines in terminal body weight were observed in both F1 generation males and females of the 6,000 ppm group. There were various organ weight changes which were statistically significant different from controls (kidneys, testes, epididymis, spleen, thyroid). Those findings were considered to be unrelated to treatment as most of those statistically different organ weights fell within their respective weight ranges from each of the study´s concurrent control groups, or were not correlated with dose and/or were correlated with a lower terminal body weight with no effects on relative organ weight.

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Table5.6.1-8: Mean absolute (g) and relative (%) organ weights

Parameter	Dose Group (ppm)							
	0		250		1,000		6,000	
F1 generation males								
Final body weight	424.0		442.0		428.9		397.3	*
Kidney weight, absolute -left	1.470		1.410		1.322	*	1.313	*
Kidney weight, absolute -right	1.519		1.480		1.419	*k	1.347	*k
Kidney weight, relative -left	0.347		0.320	*k	0.310	*k	0.332	*k
Kidney weight, relative -right	0.360		0.336	*	0.331	*	0.340	
P generation females								
Final body weight	285.4		284.5		284.7		287.9	
Kidney weight, absolute -left	1.088		1.053		1.044		1.038	
Kidney weight, absolute -right	1.141		1.091		1.110		1.076	
Kidney weight, relative -left	0.382		0.371		0.369		0.361	*
Kidney weight, relative -right	0.400		0.384		0.392		0.374	*
F1 generation females								
Final body weight	268.4		269.7		261.8		252.8	*
Kidney weight, absolute -left	1.081		1.023		1.008	*	0.949	*
Kidney weight, absolute -right	1.136		1.092		1.051	*	1.011	*
Kidney weight, relative -left	0.404		0.379	*	0.385		0.374	*
Kidney weight, relative -right	0.424		0.405		0.401	*	0.399	*

* = $p < 0.05$ (ANOVA+Dunnett`s test); *k = $p < 0.05$ (Kruskal-Wallis ANOVA+Mann-Whitney U-test)

Histopathology: Histopathology findings considered to be compound-related were confined to the kidneys of the F1-generation male and female rats of the 6,000-ppm group and the epididymis of a single F1 male from the 6,000-ppm dose group.

In F1-generation male and female rats of the 6,000 ppm group, a minimal to moderate multifocal tubular dilatation was observed in the outer portion of the medulla, containing occasional proteinaceous material. In general, both kidneys were affected with the severity and incidence being greater in males.

The epididymis of one single F1-generation male rat (animal number: SJ3514) had a moderate number of abnormal sperm within the tubules. Although microscopically there was only one animal with abnormal sperm, the sperm were similar in appearance (amorphous sperm heads) to abnormal sperm observed microscopically in the males of the 6,000-ppm F1 eight-to-nine week old interim animals and the 10,000 ppm P males in the Dose Range-Finding Study (Report No. 201300-1).

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No treatment-related findings were observed in the reproductive tract of females of either generation (ovaries, oviducts, uterus, cervix, vagina,). Ovarian follicles were counted from control and 6,000-ppm dose F1-generation females. There was no difference in the number of primordial follicles, antral follicles, or corpora lutea in the 6,000-ppm females, when compared to controls.

Table 5.6.1-9: Incidence of treatment-related changes in the kidneys

	Dose group (ppm)			
	0	**250**	**1,000**	**6,000**
F1 generation males				
Number of animals examined	**30**	**30**	**30**	**30**
Kidney, tubular dilatation				
minimal	**0**	**0**	**0**	**13**
slight	**0**	**0**	**0**	**5**
moderate	**0**	**0**	**0**	**5**
total affected	**0**	**0**	**0**	**23**
F1 generation females				
Number of animals examined	**30**	**30**	**30**	**30**
Kidney, tubular dilatation				
minimal	**0**	**0**	**0**	**7**
slight	**0**	**0**	**0**	**8**
moderate	**0**	**0**	**0**	**0**
total affected	**0**	**0**	**0**	**15**

b) Reproductive function: Estrus cycle staging did not indicate treatment-related effects on mean cycle length and number of normally cycling females in both P- and F1-generation females at any dietary level tested.

In P-generation males, sperm motility (percent motile and percent progression) and epididymal sperm count was evaluated for the controls and all treatment levels. Morphology and testicular counts were evaluated for the controls and 6,000 ppm dose group. There were no compound-related effects on any sperm parameter at any dietary level. Statistical differences observed on epididymal counts for the 1,000- and 6,000-ppm males are not considered to be compound-related, based on the following. 1) There were no effects on testicular weight or testicular sperm count; 2) there was no micropathology observed in the testes, epididymis or vas deferens; 3) similar findings were not observed in the second generation.

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In F1-generation males, sperm motility (percent motile and percent progression), total sperm count (epididymal and testicular), and morphology was evaluated at all dietary levels. A morphologic effect on sperm, presenting as amorphous sperm heads, was noted and is consistent with what was seen in the one-generation study with BYI 08330 (Report No. 201300-1). Variation in susceptibility to this finding was observed as 9/30 males exhibited a minimal effect (one to four amorphous sperm heads were noted out of 200 viewed) but only one outlying male (No. 3514) was affected to the extent that it compromised fertilizing capabilities for this one animal (i.e. the male presented 82% total abnormal sperm with 77% of these sperm presenting as amorphous heads) and the female found sperm positive from this male did not become pregnant. This same male showed abnormal sperm in the epididymis (see histopathology). Overall fertility in this dose group was not affected. There were no compound-related effects on sperm motility or total sperm count (epididymal or testicular) observed at any dietary level tested.

Table 5.6.1-10: Mean sperm measures

Sperm Analysis	Dose Group (ppm)							
	0		250		1,000		6,000	
P generation males								
Motility: % motile	**83.7**		**82.8**		**83.9**		**85.0**	
Motility: % progressive	**58.8**		**59.0**		**60.6**		**61.7**	
Sperm counts: sperm/g testis	**89.95**		**N/A**		**N/A**		**87.33**	
Sperm counts: sperm/g epididymis	**622.95**		**562.85**		**481.82**	*	**472.27**	*
Morphol.: number of normal sperm cells	**197.3**		**N/A**		**N/A**		**196.0**	
Morphol.: number of abnormal sperm cells	**2.0**		**N/A**		**N/A**		**2.8**	
Morphol: number with detached heads	**0.8**		**N/A**		**N/A**		**1.2**	
F1 generation males								
Motility: % motile	**81.1**		**82.8**		**83.9**		**79.9**	
Motility: % progressive	**56.6**		**59.7**		**59.3**		**55.7**	
Sperm counts: sperm/g testis	**87.8**		**90.3**		**89.9**		**79.3**	
Sperm counts: sperm/g epididymis	**517.2**		**490.3**		**520.1**		**496.5**	
Morphol.: number of normal sperm cells	**196.3**		**196.8**		**197.3**		**189.9**	
Morphol.: number of abnormal sperm cells	**2.9**		**2.6**		**1.9**		**8.9**	
Morphol: number with detached heads	**0.8**		**0.6**		**0.9**		**1.3**	

* = $p < 0.05$

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c) Reproductive performance: Overall reproductive performance was not effected on any parameter measured (e.g., mating, fertility or gestation indices, days to insemination, gestation length, or the median number of implants) in either generation at any dietary level tested. Comparison of the number of uterine implantation sites at necropsy with the number of delivered pups revealed the absence of any treatment-related effect on prenatal loss in both generations.
The litter parameters determined at birth (pup weight at birth, total number of pups born, stillborn pups, live birth index, percentages of male pups born, mean litter size at birth) were not affected in both generations.

d) Neonatal toxicity

General observations: There were no treatment-related effects on the viability parameters of the pups (viability index, lactation index), and no clinical signs and no malformations were observed in both F1 and F2 pups. Pup weights at birth were not affected (F1 and F2).
On lactation day 21, the F1 pup body weights for both sexes in the 6,000 ppm group was significantly lower than control (8.2%). The pup body weight gains for the 6,000-ppm group was statistically decreased (9.1%) during days 4-21 of lactation. These pup weight declines are considered to be secondary to the maternal declines in food consumption observed during the lactation period as well as the possibility that the pups themselves may not be eating the food.
On lactation days 14 and 21, the F2 pup body weights for both sexes in the 6,000-ppm group were significantly less than control (7.5% and 10.3%, respectively). The pup body weight gains for the 6,000-ppm group were statistically decreased (12.0%) during days 4-21 of lactation. These pup weight declines corresponded with maternal body weight and food consumption declines.

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Table 5.6.1-11: Mean pup body weights (g)

	Dose Group (ppm)							
	0		250		1,000		6,000	
F1 litters								
Body weight – LD 0	5.8		5.8		5.7		5.7	
Body weight – LD 4 [a]	9.1		9.3		9.2		8.7	
Body weight – LD 4 [b]	9.1		9.3		9.2		8.7	
Body weight – LD 7	14.6		14.9		14.8		13.9	
Body weight – LD 14	30.0		30.7		30.4		28.2	
Body weight – LD 21	45.2		47.1		45.9		41.5	**
Weight gain – LD 0-21	39.4		41.2		40.2		35.8	**
F2 litters								
Body weight – LD 0	5.8		5.6		5.8		5.8	
Body weight – LD 4 [a]	9.8		9.3		9.5		9.6	
Body weight – LD 4 [b]	9.8		9.3		9.5		9.6	
Body weight – LD 7	15.5		14.9		15.2		14.8	
Body weight – LD 14	30.8		30.5		31.0		28.5	**
Body weight – LD 21	46.6		45.8		45.5		41.8	**
Weight gain – LD 0-21	40.8		40.2		39.7		35.9	**

a = before standardization (culling); b = after standardization (culling); ** = $p < 0.01$

Gross pathology, organ weights: In F1 and F2 weanlings no treatment-related macroscopical alterations were observed at any dietary level tested.
Secondarily to the decreased terminal body weights at 6,000 ppm in male (F1, F2,) and female (F1, F2) weanlings, absolute brain weights were decreased (F2 males, F2 combined sexes) and relative brain weights were increased (F1 and F2 males and females; F1 and F2 combined sexes) at 6,000 ppm; changes in brain weights are therefore not interpreted as direct adverse effects on this organ.
At 6,000 ppm, absolute spleen weights were decreased in combined F1 weanlings and in F2 male and female weanlings. F2 males and females also had a decreased absolute thymus weight at 6,000 ppm. The decrease in absolute spleen and thymus weight was considered a reflection of the low mean body weight of the 6,000 ppm pup dose group, and are therefore not interpreted as direct adverse effects on these organs.

Table 5.6.1-12: Mean absolute (g) and relative (%) organ weights of weanlings

Parameter	Dose Group (ppm)							
	0		250		1,000		6,000	
F1 male+female weanlings (combined)								
Brain weight, absolute	1.454		1.449		1.465		1.421	
Brain weight, relative	3.252		3.096		3.231		3.444	**
Spleen weight, absolute	0.205		0.229		0.219		0.185	*
Spleen weight, relative	0.454		0.483		0.476		0.445	
F2 male+female weanlings (combined)								
Brain weight, absolute	1.479		1.467		1.474		1.444	*
Brain weight, relative	3.194		3.233		3.274		3.491	**
Spleen weight, absolute	0.200		0.204		0.203		0.160	**
Spleen weight, relative	0.430		0.445		0.443		0.382	**
Thymus weight, absolute	0.214		0.207		0.202		0.187	**
Thymus weight, relative	0.459		0.453		0.445		0.450	

* = $p < 0.05$; ** = $p < 0.01$

Histopathology: There were no compound-related microscopic findings observed in F1 and F2 weanlings of either sex at any dietary level tested.

Developmental milestones: Maturation of external sexual organs (balanopreputial separation, vaginal opening) was not affected in F1 weanlings. Anogenital distance was not triggered to be performed in this study.

III. Conclusion: The NOAEL for male parental toxicity was established at 1,000 ppm (equal to premating doses of 70.7 mg/kg bw/day or 79.5 mg/kg bw/day in P- or F1-generation males, respectively), based on body weight gain declines in P-generation males at 6,000 ppm (equal to 419.3 mg/kg bw/day) and body weight gain declines, decreased terminal body weights and increased renal multifocal tubular dilatation in F1-generation males at 6,000 ppm (equal to 486.7 mg/kg bw/day). The highest dietary exposure level (6,000 ppm) caused slight effects on sperm cell morphology in the F1 generation males.

The NOAEL for female parental toxicity was established at 1,000 ppm (equal to premating doses of 82.5 mg/kg bw/day or 90.3 mg/kg bw/day in P- or F1-generation females, respectively), based on reduced food consumption during lactation in P-generation females at 6,000 ppm(equal to 484.7 mg/kg bw/day) and reduced food consumption during lactation, reduced body weights (end premating), body weight gain declines (premating), decreased terminal body weights and increased renal multifocal tubular dilatation in F1-generation females at 6,000 ppm (equal to 539.5 mg/kg bw/day).

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The NOAEL for reproductive toxicity was established at 1,000 ppm in males (equal to premating doses of 79.5 mg/kg bw/day in F1 generation males), based on abnormal sperm cell morphology in F1-generation males at 6,000 ppm (equal to 486.7 mg/kg bw/day), which included one animal with abnormal epididymal sperm cells and pronounced abnormal spermatozoa leading to compromised fertilizing capabilities and another few animals (9/30) showing one to four amorphous sperm heads out of 200 sperm cells viewed. Reproductive performance was not impaired at 6,000 ppm in either generation.

The NOAEL for reproductive toxicity was established at 6,000 ppm in females (equal to premating doses of 484.7 mg/kg bw/day or 539.5 mg/kg bw/day in P- or F1-generation females, respectively), based on the absence of effects at 6,000 ppm on mean estrus cycle length, number of normally cycling females, mating-, fertility- and gestation indices, mean gestation duration, mating performance, vaginal opening, litter parameters determined at birth (pup weight, total number of pups born, stillborn pups, viability index on lactation day 0, sex ratio, mean litter size).

The NOAEL for neonatal toxicity was established at 1,000 ppm, based on decreased body weights on lactation day 21 and decreased body weight gain in F1 pups and decreased body weights on lactation days 14 and 21 and decreased body weight gain in F2 pups at 6,000 ppm. Pup weight declines corresponded with maternal body weight and food consumption declines.

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IIA 5.6.2 Separate male and female studies

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IIA 5.6.3 Three segment designs

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IIA 5.6.4 Dominant lethal assay for the male fertility

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IIA 5.6.5 Cross-matings of treated males with untreated females and *vice versa*

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IIA 5.6.6 Effects on spermatogenesis

Mechanistic study for identification/characterization of time of onset and location of injury to the testis is listed under IIA 5.5.4.

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IIA 5.6.7 Effects on oogenesis

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IIA 5.6.8 Sperm motility, mobility and morphology

Included in IIA 5.6.1

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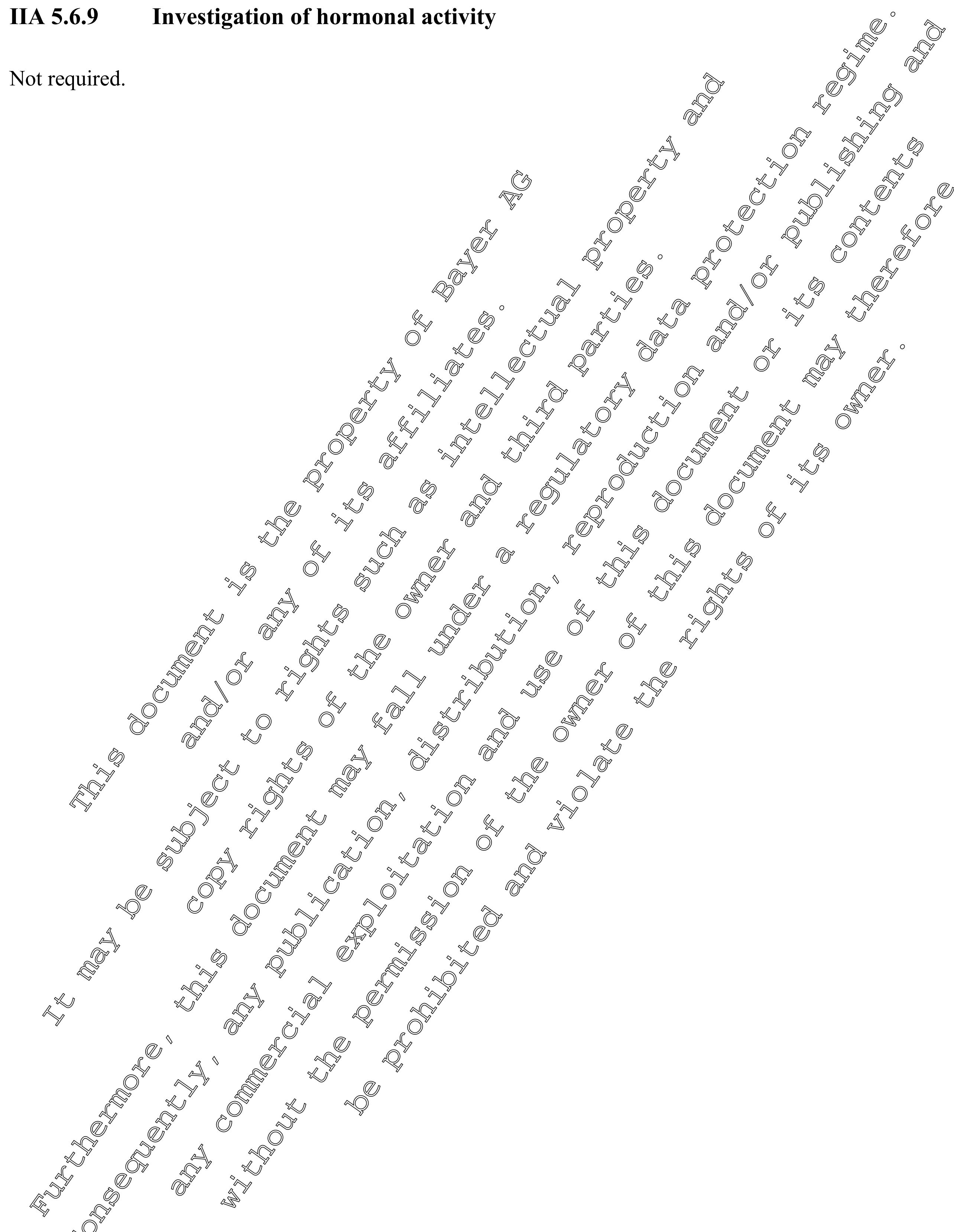
IIA 5.6.9 Investigation of hormonal activity

Not required.



IIA 5.6.10 Teratogenicity test by the oral route in the rat

Report:	**KIIA 5.6.10/01, [redacted]; 2001**
Title:	BYI 08330 – Pilot study on developmental toxicity in rats after oral administration
Report No &	T3068559
Document No	M-021476-01-2
Guidelines:	Not required
OECD/FIFRA	No
GLPS	

Executive Summary

In the present pilot developmental toxicity study, five groups of 7 inseminated Wistar (Hsd Cpb:WU) rats each were treated daily, orally by gavage, with BYI 08330, batch no. NLL 6425-5, purity: 98,8%, suspended in 0.5% aqueous carboxymethylcellulose, from day 6 to day 19 post coitum (p.c.) at doses of 0, 50, 200, 800 and 1,000 mg/kg bw/day. For better clarification of toxic effects, 7 further females were added later on to the 1,000 mg/kg dose group. The fetuses were delivered by cesarean section on day 20 of gestation. Investigations were performed on general tolerance of the test compound by the females as well as on its effect on intrauterine development (including external, skeletal and visceral evaluation of fetuses).
Maternal effects were noted at 800 mg/kg (body weight loss, impaired body weight development) and at 1000 mg/kg (respiratory findings, piloerection, body weight loss, impaired body weight gain, increased or decreased water intake, increased urination, light colored feces). Effects on intrauterine development could not be completely excluded at the 200 mg/kg level (possibly marginally reduced fetal weight), were evident at a dose level of 800 mg/kg (reduced placental and possibly fetal weight, retarded ossification, wavy ribs) and were clearly observed at the 1,000 mg/kg dose level (distinctly reduced fetal and placental weight, necrotic placental borders, retarded ossification, wavy and 14th ribs and possibly marginally increased incidence of common malformations, although within the upper range of historical control data).

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Results and Discussion:

Maternal toxicity:

Treatment related effects on respiration (transiently gasping or labored breathing, respiratory sounds), water intake (increased or decreased water intake at the end of treatment), excretion (increased urination and light colored feces) and appearance (piloerection) were restricted to the 1,000 mg/kg group. Slight body weight loss for one day (6 to 7 p.c.) occurred at a dose level of 800 mg/kg and above; in the 1,000 mg/kg group together with slightly reduced feed intake during treatment and more pronouced reduction at the end of treatment. Reduced carcass weight and corrected body weight gain were observed at the 800 mg/kg level (marginal effects) and to a more distinct degree in the 1,000 mg/kg group. Final body weight and body weight gain during gestation were as well distinctly reduced in the 1,000 mg/kg group.

Necropsy revealed no treatment related findings at a dose level up to 800 mg/kg and toxicological relevance was not assumed for a slight renal pelvis dilation seen in one female of the 1000 mg/kg group due to comparable findings in historical controls.

Developmental toxicity: Reproductive parameters, i.e. gestation rate, postimplantation loss, litter size and fetal sex distribution were not affected to a toxicologically relevant degree by treatment at a dose level up to and including 1,000 mg/kg. A marginal reduction of placental weight was seen at a dose level of 800 mg/kg and above; in the 1,000 mg/kg group together with increased incidence of necrotic placental borders and possibly engorged placentae. A marginal effect on fetal weight (reduction) could not be completely excluded at dose levels of 200 and 800 mg/kg and was clearly evident at the 1,000 mg/kg dose level. Final evaluation of this finding at the 200 mg/kg and 800 mg/kg dose levels was not possible in this pilot study.

Overall incidence of common unspecific malformations (dysplastic tubular bones, interatrial septal defect of the heart) was marginally increased at the distinctly maternal toxic 1,000 mg/kg dose level (i.e. 5.5% of fetuses and 35.7% of litters affected) though within the upper range of historical control data. One case each of dysplastic tubular bones was seen in the other dose groups; at the 800 mg/kg dose level together with one case of interatrial septal defect. Since these malformations are common findings in the rat strain used and their incidence lay within the range of historical control data, toxicological relevance was not assumed for these findings up to and including the 800 mg/kg dose level. Final evaluation was not possible at the 1,000 mg/kg level in this pilot study.

External and visceral fetal evaluation gave no further indication for toxicologically relevant effects up to and including 1,000 mg/kg.

Retarded fetal ossification together with marginally to slightly increased incidence of wavy ribs (variation) occurred at a dose level of 800 mg/kg and above. Incidence of additional 14th ribs (variation) was as well slightly increased at the 1,000 mg/kg level.

Conclusion: Based on these results, it is concluded that the dose level of 1,000 mg/kg bw/day is suitable for the high dose level in the main developmental toxicity study of BYI 08330 in rats. It is also considered that the dose levels of approximately 140 mg/kg bw/day and 20 mg/kg bw/day are suitable for the mid and low dose levels, respectively.

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Report:	**KIIA 5.6.10/02, ; 2004**
Title:	BYI 08330 - Developmental Toxicity Study in Rats After Oral Administration
Report No &	AT01413
Document No	M-086404-01-2
Guidelines:	OECD 414 (2001), 88/302/EEC (1988), US-EPA-OPPTS 870.3700 (1998), JMAFF 12-Nousan-No. 8147 (2000, amended 2001); Deviations: none
Deviations:	Homogeneity of the 2 mg/ml concentration was marginally out of the accepted range (RSD 11.82% versus limit value of 10%) during the 2nd content check (see page 569 of the Annex). However, this deviation lay only marginally above the accepted range, the content of this formulation was confirmed and only 7 females were treated with this formulation for 3 or 4 days at the end of gestation. Thus, an impact of this finding on the outcome of the study was excluded.
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In the present developmental toxicity study, groups of 25 inseminated Wistar (Hsd Cpb:WU) rats each were treated daily, orally by gavage, with BYI 08330, batch no. NLL 6425-14-a, purity: 99.0%, suspended in 0.5% aqueous carboxymethylcellulose, from day 6 to day 19 post coitum (p.c.) at doses of 0, 20, 140, and 1,000 mg/kg bw/day. The fetuses were delivered by cesarean section on day 20 of gestation. The doses were selected based on the results of a pilot developmental toxicity study in rats in which doses of 0, 50, 200, 800 and 1,000 mg/kg bw/day were tested. The stability and homogeneity of 1.0 and 100 mg/mL formulations, and the achieved concentrations of all formulations, were confirmed by analysis during the study.

Mortality, appearance and behavior were not affected by treatment with BYI 08330. Treatment-related effects at 1,000 mg/kg bw/day included impaired feed intake, transient marginal body weight loss, impaired body weight gain, reduced final body weight and reduced carcass weight. Necropsy revealed no treatment related findings. A marginal reduction of placental weight together with a more distinct reduction of fetal weight was observed in the 1,000 mg/kg dose group.

External, visceral and skeletal evaluation of fetuses revealed a slightly increased number of fetuses and litters with generally common unspecific malformations at the maternally toxic 1,000 mg/kg dose level though at the upper range of historical control data. A potential of BYI 08330 to induce a specific type of malformation was not deduced from these findings. Overall incidence and type of malformations at the 20 mg/kg and 140 mg/kg bw/day dose level did not indicate a treatment related effect.

Fetal external deviations were not observed in this study and incidence and type of visceral deviations of fetuses revealed no treatment-related effects at a dose level up to and including 1,000 mg/kg bw/day. Skeletal - including cartilaginous tissue - evaluation of fetuses revealed retarded ossification at the 1,000 mg/kg dose level together with an increased incidence of skeletal variations (wavy ribs, 14th ribs, combined osseous and cartilaginous findings). Furthermore, retarded ossification of single localisations and increased incidence of wavy ribs were noted in the 140 mg/kg bw/day dose group and at the 20 mg/kg dose level without clear dose relation.

Based on the results of a supplementary study with BYI 08330 (Report No. AT01512) which revealed no indication for a treatment related increase of wavy ribs at a dose level up to and including 140 mg/kg bw/day and no evidence for effects on degree of ossification at a dose level up to and

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including 140 mg/kg bw/day, treatment relationship was excluded in the present study for retarded ossification and for incidence of wavy ribs at a dose level up to and including 140 mg/kg bw/day.

The following no-observed-adverse-effect levels (NOAEL) were thus determined in this developmental toxicity study:

Systemic maternal toxicity:	140 mg/kg body weight/day
Intrauterine development:	140 mg/kg body weight/day

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I. Materials and methods

A. Materials:

1. Test Material: BYI 08330

Description:	technical, white powder, room temperature storage
Lot/Batch:	NLL 6425-14-a
Purity:	99.0% (cis-isomer)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS of TGAI:	203313-25-1

2. Vehicle and/or positive control:
0.5% carboxymethylcellulose (CMC high viscosity, by [redacted] [redacted], Switzerland) in demineralized water as a suspension

3. Test animals:

Species:	Rat	
Strain:	Wistar	
Age/weight at study initiation:	13 to 15 weeks old 202 to 247 g (day 0 post coitum)	
Source:	[redacted]	
Housing:	Individually housed in Type III Makrolon cages	
Diet:	[redacted] Mouse and Rat Maintenance Diet, Art-No.: 3883.0.15 *ad libitum*	
Water:	Tap water *ad libitum*	
Environmental conditions:	**Temperature:**	21± 2°C
	Humidity:	approx. 50%
	Air changes:	at least 10/hr
	Photoperiod:	12 hrs dark/12 hrs light
Acclimatisation:	At least 7 days	

B. Procedures and study design

1. In life dates: Start: May 14, 2002 End: August 23, 2004

2. Mating: The animals were paired by placing two females overnight into a Type III cage together with one male rat. If sperm was detected in the vaginal smear taken on the morning following mating, this day was regarded as day 0 of gestation.

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3. Animal Assignment: After insemination was ascertained, the animals were assigned to their respective study group according to a computer-generated randomization procedure.

Dose (mg/kg/day)	**0**	**20**	**140**	**1,000**
Females	25	25	25	25

4. Dose selection rationale: The dose levels used were selected according to a dose-range finding-study in rats with dose levels of 0, 50, 200, 800 and 1,000 mg/kg (study No. T3068559). Maternal effects were seen at the 800 mg/kg dose level (body weight loss, impaired body weight development) and distinct at the 1,000 mg/kg dose level (respiratory findings, piloerection, body weight loss, impaired body weight gain, in- or decreased water intake, increased urination, light colored feces). Effects on intrauterine development could not be completely excluded at the 200 mg/kg level (possibly marginally reduced fetal weight), were evident at a dose level of 800 mg/kg (reduced placental and possibly fetal weight, retarded ossification, wavy ribs) and were clearly observed at the 1,000 mg/kg dose level (distinctly reduced fetal and placental weight, necrotic placental borders, retarded ossification, wavy and 14th ribs and possibly marginally increased incidence of common malformations).

5. Dosage preparation and analysis: Prior to the start of the study, stability of the test substance in aqueous 0.5% carboxymethyl-cellulose was evaluated for a period of 8 days at room temperature. Concentration and homogeneity (top, middle, and bottom) of the test mixture were evaluated once prior to the study and once during the study. Investigations on the stability and homogeneity and a content check of the active ingredient in samples of 1 mg/ml and 100 mg/ml were performed before the start of this study and covered the concentrations used in this study. Data on stability and homogeneity were stored under the study number E5010806. Two content checks of the formulations of all concentrations as well as checks on homogeneity of the low and high concentrations were carried out during the in-life period of the study (formulations prepared May 22, and June 20, 2002, respectively). The first content check of the formulations of all concentrations as well as checks on homogeneity of the low and high concentrations revealed no significant deviation of the active ingredient content from the nominal value in the formulations of any of the treatment groups and homogeneity of the low and high concentrations also complied. Homogeneity of the 2 mg/ml concentration was marginally out of the accepted range (RSD 11.82% versus limit value of 10%) during the 2nd content check (see page 569 of the report). However, this deviation lay only marginally above the accepted range, the content of this formulation was confirmed and only 7 females were treated with this formulation for 3 or 4 days at the end of gestation. Thus, an impact of this finding on the outcome of the study was excluded, (for results see pages 568 to 569 of the report).

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Results:

Homogeneity Analysis:
The analytical data verify (see table) that the test substance was homogeneously distributed in the 1 and 100 mg/ml formulation (Suspensions had to be stirred to achieve homogenization).

Nominal Value (mg/ml)	Content (mg/ml)	Content as % of nominal value	%RSD
1	0.952	95	0.31
100	106.249	106	4.76

Stability Analysis:
The analytical data verify (see table) that the 1 and 100 mg/ml formulations were stable at room temperature for at least 8 days.

Nominal Value (mg/ml)	Content in %		Content as % of Nominal Value	
	Start	After 8 Days	Start	After 8 days
1	0.952	0.927	95	93
100	106.249	107.642	106	108

Concentration Analysis:
The analytical data verify that the test substance was homogeneously distributed in the 2 and 100 mg/ml formulations and that the contents were within defined limits.

Date of formulation preparation – May 22, 2002

Nominal Value (mg/ml)	Content (mg/ml)	Content as % of nominal value	%RSD
2	1.756	88	3.14
14	14.094	101	Content only
100	102.277	102	1.52

Date of formulation preparation – June 20, 2002

Nominal Value (mg/ml)	Content (mg/ml)	Content as % of nominal value	%RSD
2	1.822	91	11.82*
14	13.659	98	Content only
100	109.999	110	4.43

*Value not within defined limits

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6. Dosage administration: The females were treated once daily by gavage between 06:00 and 12:30 a.m. from gestation days 6 through 19 post coitum. The females of the control group received vehicle only (0.5% aqueous CMC) at the same volume. Dosing was based on the body weight from the most recent body weight determination, using a uniform dose volume of 10 ml/kg body weight.

C. Observations

1. Maternal Observations and Evaluations: The animals were checked for mortality or clinical signs at least once daily. Body weight was recorded on gestation days 0 and daily between days 6 and 20. Food consumption was measured on gestation days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, and 18-20. Dams were sacrificed on day 20 of gestation. Examinations at sacrifice consisted of a gross pathological evaluation. Each uterus was weighed and dissected. The number of implantations was determined along with early resorptions, late resorptions, and dead fetuses. The number of corpora lutea in the ovaries was recorded as well.

2. Fetal Evaluations: The number of live fetuses was noted and their sex and body weight were recorded. All of the fetuses were evaluated for external abnormalities. Every other fetus was assigned to either the visceral or skeletal examinations. In the visceral examination, the fetus was sectioned according to the modified Wilson technique and the organs in the thoracic, abdominal and pelvic regions were evaluated for abnormalities. The fetuses included in the skeletal examination were eviscerated, treated with the cartilage staining technique of Inouye, using alcian blue GX, cleared with a dilute potassium hydroxide solution and stained with Alizarin red S.

D. Data analysis

1. Statistical analyses: The following parameters were analyzed using ANOVA with pairwise comparison for statistical significance by the Dunnett's test: food consumption, body weight, uterine weight, number of corpora lutea per female, number of implantations per female, number of live fetuses per female and as a percentage of implantations per female, placental weights per female and fetal weights per female. The Chi squared test was used to evaluate the number of fetuses or litters with visceral or cartilaginous tissue observations. The Chi square test was used to evaluate as well fertility and gestation rates, number of preimplantation losses per group, number of preimplantation losses, early resorptions, late resorptions or dead fetuses per group, the number of live fetuses per group in percent of implantations, number of male or female fetuses or fetuses with undeterminable sex per group, number of fetuses or litters with external, visceral or skeletal findings, and number of fetuses or litters with malformations. If a significant difference existed, Fisher's exact test with the Bonferroni correction was used for pairwise comparison. The Kruskal-Wallis test was used to evaluate the number of preimplantation losses per female, the number of postimplantation losses, early resorptions, late resorptions, or dead fetuses per female, number of male or female fetuses or fetuses of undeterminable sex per female, and the proportion of placental, fetal external or visceral findings per female. If a significant difference existed, Dunn's test was used for pairwise comparisons.

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2. Historical control data: Historical control data were provided to allow comparison with concurrent study data. Control animal data from studies conducted between 1987 and 2003 were included in this database.

II. Results

Findings:

Maternal toxicity: Mortality, appearance and behavior were not affected by treatment with BYI 08330. Feed intake at 1,000 mg/kg was significantly reduced by 19%, 10%, 9% and 11% relative to controls on days 6 – 9, day 9 – 12, day 15 – 18 and day 18 – 20 p.c., respectively. No treatment-related effect occurred at lower levels.

Table 5.6.10-1: Mean feed intakes (g/animal/day) of pregnant animals

Parameter	Dose Group (mg/kg bw/day)							
	0		20		140		1,000	
Feed intake								
day 0 - 3	**20.35**		**20.51**		**20.41**		**19.56**	
day 3 - 6	**21.00**		**21.21**		**20.81**		**19.94**	
day 6 - 9	**21.37**		**21.06**		**20.62**		**17.32**	**
day 9 -12	**22.02**		**21.81**		**21.36**		**19.80**	*
day 12 - 15	**22.48**		**22.61**		**21.43**		**20.64**	
day 15 – 18	**25.85**		**26.04**		**24.78**		**23.53**	**
day 18 – 20	**26.42**		**25.73**		**25.83**		**23.59**	**

* = $p < 0.05$; ** = $p < 0.01$

Transient body weight loss occurred on day 7 in dams treated at 1,000 mg/kg bw/day, and weight gain was significantly reduced during gestation by approximately 20% at 1,000 mg/kg bw/day. Corrected body weight gain at 1,000 mg/kg bw/day was also significantly reduced by 29% relative to controls. At the 140 mg/kg bw/day dose level body weight loss was not observed, but body weight gain during the treatment (day 6 to 19 p.c.; with statistical significance) and overall gestation period (day 0 to 20 p.c.) was reduced which resulted as well in statistically significantly reduced final body weights. These findings were possibly both related to incidentally lower litter size in the 140 mg/kg bw/day dose group and to incidental morbidity of female no. 78 and are considered not treatment related, as effects on carcass weight and corrected body weight gain were not evident in the 140 mg/kg bw/day dose group and statistical significance was not longer evident for final body weights when this female was excluded from calculations.

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Furthermore, a supplementary oral developmental toxicity study with BYI 08330 in Wistar rats (report no. AT01512) at dose levels of 0, 10, 35 and 140 mg/kg bw/day revealed no effects on body weight development at dosages up to and including 140 mg/kg bw/day. Thus a treatment related effect on body weight development was not assumed at the 140 mg/kg bw/day dose level. Necropsy revealed no treatment related findings at a dose level up to and including 1,000 mg/kg bw/day.

Table 5.6.10-2: Mean body weights or body weight gains (g) of pregnant animals

Parameter	Dose Group (mg/kg bw/day)							
	0		20		140		1,000	
Final body weight on day 20	**343.9**		**337.3**		**327.7**	*	**319.9**	**
Absolute body weight gain on day 6 - 19	**86.8**		**85.2**		**74.8**	*	**69.5**	**
Absolute body weight gain on day 0 - 20	**123.4**		**121.0**		**111.1**		**100.3**	**
Corrected body weight gain on day 0 – 20	**50.3**		**46.9**		**47.3**		**35.6**	**

* = p < 0.05; ** = p < 0.01

Developmental toxicity: No effects occurred on gestation rate, postimplantation loss, number of fetuses, sex distribution, and placental appearance. A marginal reduction of placental weight together with a more distinct reduction of fetal weight was observed in the 1,000 mg/kg dose group. The marginally lower fetal weight of the 140 mg/kg bw/day group was considered incidental and not related to treatment. Furthermore, effects on fetal weight were not evident in a supplementary oral developmental toxicity study with BYI 08330 (T7063008). Here mean fetal weights in the control, 10 mg/kg, 35 mg/kg and 140 mg/kg bw/day dose groups were 3.60, 3.61, 3.57 and 3.60 grams, respectively which renders a treatment related effect at the 140 mg/kg bw/day dose level in the present study unlikely.

Table 5.6.10-3: Intrauterine development

Parameter	Dose Group (mg/kg bw/day)							
	0		20		140		1,000	
Placental weight in g	**0.62**		**0.64**		**0.65**		**0.57**	
Mean number of fetuses/litter	**12.4**		**12.5**		**11.0**		**12.3**	
Postimplantation loss	**0.6**		**0.5**		**1.0**		**0.7**	
Males in %	**54.2**		**47.7**		**47.9**		**48.0**	
Fetal weight in g	**3.63**		**3.58**		**3.50**		**3.12**	**

** = p < 0.01

Fetal external deviations were not observed in this study and the incidence and type of visceral deviations of fetuses was not affected up to and including 1,000 mg/kg bw/day. Skeletal evaluation revealed a slightly increased number of fetuses and litters with generally common unspecific malformations (4.4% and 40.9%, respectively) at the maternally toxic 1,000 mg/kg dose level.

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However, both the fetal and litter incidences of malformations were within or at the upper limit of the historical control incidence range (up to 6.9% of fetuses and 40.0% of litters).
Malformations at the 1,000 mg/kg dose level included one case of cleft palate, another fetus with co-arctation of aortic arch as well as common unspecific malformations (one case of microphthalmia, one fetus with atrial septal defect of the heart, 4 fetuses in 4 litters with dysplastic forelimb bones, one fetus with a supernumerary lumbar vertebra and 3 fetuses within 3 litters with altered appearance of sacral vertebral arches and pelvic shift). Although two of the findings are rare observations (cleft palate, co-arctation of aortic arch), a specific potential of BYI 08330 to induce these types of malformations was not assumed since these findings occurred only once in the 1,000 mg/kg group, were as well seen spontaneously in control or low dose groups of recent developmental toxicity studies and were different in type.

Table 5.6.10-4: Summary of fetal malformations

Parameters	Dose Group (mg/kg bw/day)			
	0	20	140	1,000
Cleft palate				1
Microphthalmia, unilateral	1	1		1
Anophthalmia, unilateral	1	1		
Upper jaw shortened, macroglossia, domed head, dysplasia fore- and hindlimbs, skull and vertebral column			1	
Lobe of thyroid gland absent	3(2)		1	
Atrial septal defect of the heart	1	1		1
Co-arctation of aortic arch between left carotid and left subclavian arteries, ascending aorta reduced in size, left subclavian artery arises from descending aorta				1
Dysplasia of forelimb bones (scapula, humerus, radius and/or ulna)	1	2(2)		4(4)
Supernumerary lumbar vertebra				1
1st sacral vertebral arch shaped like lumbar vertebral arch, cartilaginous part not fused with processus transversus of the 2nd sacral vertebral arch, pelvis shifted caudally				3(3)
Number of fetuses per group	247	301	253	270
Number of fetuses with malformations	7	5	2	12
Malformed fetuses per group (%)	2.83	1.66	0.79	4.44
Number of litters per group	20	24	23	22
Number of litters with malformations	4	4	2	9
Malformed litters per group (%)	20.0	16.7	8.7	40.9

( ) number of litters affected

Statistically significantly retarded ossification in various locations (phalanges, sternebrae, vertebrae, skull bones) were noted at the 1,000 mg/kg dose level in the osseus and/or cartilagineous parts. Increased incidences of skeletal variations (wavy ribs, 14th ribs, combined osseous and cartilagineous

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findings) were seen as well. All these findings can be ascribed to the observed growth retardation at this dose level and are considered to be an indirect effect of treatment.

Retarded ossification was also noted in the 140 mg/kg bw/day dose group and in the 20 mg/kg dose group on a fetal basis at single bones of the fore- and hindpaws without clear dose relationship (140 mg/kg bw/day: distal phalanx of 4th digit left, distal phalanx of 1st and 4th toe right and 1st to 4th toe left; 20 mg/kg: distal phalanges of 1st to 4th digit right and 1st and 3rd left, proximal phalanx of 4th digit right, metacarpal of 5th digit right) together with incomplete ossification of the 6th sternal segment and the supraoccipital bone (at 20 mg/kg only). None of these findings were statistically significantly different on a litter basis and all findings were within the range of historical control values. Furthermore, the changes of the low and mid dose groups were poorly correlated with dose. In addition, a supplemental study (Report No. AT01512) revealed no relevant effects on the degree of ossification at dose levels of 10, 35 and 140 mg/kg bw/day. Moreover, no statistically significant changes were noted in ossification means of all but two of the respective bones up to and including 140 mg/kg bw/day and this significance was only seen on a fetal basis. Based on the clarifying results of the supplementary study, a treatment-related effect for retarded ossification in the present study at doses of 20 and 140 mg/kg bw/day is considered unlikely.
The number of individual ribs with wavy appearance and sum of wavy ribs was statistically significantly increased on a fetal basis in the 140 and 20 mg/kg dose group, but not on a litter basis (see table below). The fetal incidences at 20 and 140 mg/kg bw/day for this skeletal variant were slightly outside the historical control incidence ranges of 2.7 – 15.1% (historical control range derived from 22 studies performed between 1996 and 2001). However, the mean fetal control incidence was rather low and a dose-response relationship was not clearly evident for the mid- and low dose group, assuming that the statistical differences noted for wavy ribs were apparently due to chance variation. Based on the clarifying results of the supplementary study, which revealed no indications for a treatment related increase of wavy ribs at dose levels of 10, 35 and 140 mg/kg bw/day, a treatment-related effect for the occurrence of wavy ribs in the present study at doses of 20 and 140 mg/kg bw/day is excluded.

Table 5.6.10-5: Summary of fetal skeletal incidences

Parameter	Fetal and litter incidences (%) per Dose Group (mg/kg bw/day)							
	0		20		140		1,000	
Number of fetuses evaluated	**127**		**159**		**135**		**146**	
Wavy ribs (sum); fetal incidence	**5.5**		**17.0**	**	**17.8**	**	**57.5**	**
14th ribs (sum); fetal incidence	**15.0**		**10.7**		**12.6**		**45.9**	**
Number of litters evaluated	**20**		**24**		**23**		**22**	
Wavy ribs (sum); litter incidence	**30.0**		**41.7**		**52.2**		**95.5**	**
14th ribs (sum); litter incidence	**50.0**		**50.0**		**47.8**		**86.4**	

** = $p < 0.01$

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Conclusion: The NOAEL for maternal toxicity was 140 mg/kg bw/day, based on reduced feed intake, transient body weight loss, reduced terminal body weight, and reduced corrected body weight gain at 1,000 mg/kg bw/day. The NOAEL for developmental toxicity was established at 140 mg/kg bw/day bw/day, based on a marginal reduction of placental weight, distinctly decreased fetal weights, slightly increased incidence of common unspecific malformations, increased incidence of skeletal variations (wavy ribs, 14th ribs, combined osseous and cartilaginous findings), and increased incidence of retarded ossification at 1,000 mg/kg bw/day. No evidence for a primary embryotoxic or teratogenic potential of BYI 08330 was determined.

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Report:	**KIIA 5.6.10/03, [redacted]; 2004**
Title:	BYI 08330 - Supplementary Developmental Toxicity Study in Rats After Oral Administration
Report No & Document No	AT01512 M-091750-01-2
Guidelines:	OECD 414 (2001), 88/302/EEC (1988), US-EPA-OPPTS 870.3700 (1998), JMAFF 12-Nousan-No. 8147 (2000, amended 2001); Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

Based on the results of the main developmental toxicity study with BYI 08330 in rats (Report No. AT01413) with dose levels of 20, 140 and 1,000 mg/kg bw/day where equivocal retarded ossification of single localisations and equivocal increased incidence of wavy ribs were noted in the 140 and in the 20 mg/kg dose group without clear dose relation, a supplementary developmental toxicity study was performed for clarification of results.

Groups of 25 inseminated Wistar (Hsd Cpb:WU) rats each were treated daily, orally by gavage, with BYI 08330, batch no. NLL 6425-14-a, purity: 99.1%, suspended in 0.5% aqueous carboxymethylcellulose, from day 6 to day 19 post coitum (p.c.) at doses of 0, 10, 35, and 140 mg/kg bw/day. The fetuses were delivered by cesarean section on day 20 of gestation. The stability and homogeneity of 1.0 and 100mg/mL formulations, and the achieved concentrations of all formulations, were confirmed by analysis during the study. The study methodology was identical to the former study with the following exceptions: The liver of the dams were weighed and subjected to histopathology. Blood samples were collected from maternal animals at necropsy for measurement of red blood cell parameters (Erythrocytes, hemoglobin, hematocrit, MCV, MCH and MCHC) and clinical chemistry parameters (ASAT, ALAT, ALP, GGT, CHOL, TG, CREA, UREA, Ca, P).
No treatment related maternal effects were observed at any dose level with respect to mortality, clinical signs, feed intake, body weight development, hematology and clinical chemistry parameters, liver weight, necropsy findings and histopathology of the liver. There were also no treatment related effects at any dose level on reproductive parameters, (i.e. gestation rate, postimplantation loss, litter size, placental weight and appearance, fetal weight and fetal sex distribution). The incidence and type of fetal malformations were unaffected by treatment at levels up to and including 140 mg/kg bw/day. Meaningful fetal external or visceral deviations were not evident at a dose level up to and including 140 mg/kg bw/day in this study.
Fetal skeletal ossification and incidence of skeletal variations including evaluation of cartilaginous structures revealed no evidence for treatment related effects at a dose level up to and including 140 mg/kg bw/day. The incidence of wavy ribs was not affected at a dose level up to and including 140 mg/kg bw/day.
Thus the following no-observed-adverse-effect levels (NOAEL) were determined:
Maternal toxicity: 140 mg/kg bw/day
Developmental toxicity: 140 mg/kg bw/day

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I. Materials and methods

A. Materials:

1. Test Material: BYI 08330

Description:	technical, white powder, room temp storage
Lot/Batch No.:	NLL 6425-14-a
Purity:	99.1%. (cis-isomer)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No.of TGAI:	203313-25-1

2. Vehicle and/or positive control: 0.5% carboxymethylcellulose (CMC high viscosity, by Fluka , Switzerland) in demineralized water as a suspension

3. Test animals:

Species:	Rat	
Strain:	Wistar	
Age/weight at study initiation:	13 to 15 wks old 202 to 247 g (day 0 post coitum)	
Source:		
Housing:	Individually housed in Type III Makrolon cages	
Diet:	Mouse and Rat Maintenance Diet, Art-No.: 3883.0.15 *ad libitum*	
Water:	Tap water *ad libitum*	
Environmental conditions:	**Temperature:**	22 ± 2ºC
	Humidity:	approx. 50%
	Air changes:	at least 10/hr
	Photoperiod:	12 hrs dark/12 hrs light
Acclimatisation:	At least 7 days	

B. Procedures and study design

1. In life dates: Start: February 5, 2003 End: October 7, 2004

2. Mating: The animals were paired by placing two females overnight into a Type III cage together with one male rat. If sperm was detected in the vaginal smear taken on the morning following mating, this day was regarded as day 0 of gestation.

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3. Animal Assignment: After insemination was ascertained, the animals were assigned to their respective study group according to a computer-generated randomization procedure.

Dose (mg/kg bw/day)	0	10	35	140
Females	25	25	25	25

4. Dose selection rationale: The dose levels used were selected based on the results of a preceding guideline study in rats with dose levels of 0, 20, 140 and 1,000 mg/kg bw/day (see Report No. AT01413) where a clear no-observed-adverse-effect-level (NOAEL) could not be established with respect to the egree of fetal ossification and incidence of wavy ribs.

5. Dosage preparation and analysis: Prior to the start of the study, stability of the test substance in aqueous 0.5% carboxymethyl-cellulose was evaluated for a period of 8 days at room temperature. Concentration and homogeneity (top, middle, and bottom) of the test mixture were evaluated once prior to the study and once during the study. Investigations on the stability and homogeneity and a content check of the active ingredient in samples of 1 mg/ml and 100 mg/ml were performed before the start of this study and covered the concentrations used in this study. Data on stability and homogeneity were stored under the study number F5010806. Two content checks of the formulations of all concentrations as well as checks on homogeneity of the low and high concentrations were carried out during the in-life period of the study (formulations prepared May 22, and June 20, 2002, respectively). The first content check of the formulations of all concentrations as well as checks on homogeneity of the low and high concentrations revealed no significant deviation of the active ingredient content from the nominal value in the formulations of any of the treatment groups and homogeneity of the low and high concentrations also complied. Homogeneity of the 2 mg/ml concentration was marginally out of the accepted range (RSD 11.82% versus limit value of 10%) during the 2nd content check (see page 569 of the Report No. AT01413). However, this deviation lay only marginally above the accepted range, the content of this formulation was confirmed and only 7 females were treated with this formulation for 3 or 4 days at the end of gestation.

Results:

Homogeneity Analysis:

The analytical data verify (see table) that the test substance was homogeneously distributed in the 1 and 100 mg/ml formulation (Suspensions had to be stirred to achieve homogenization)

Nominal Value (mg/ml)	Content (mg/ml)	Content as % of nominal value	%RSD
1	0.952	95	0.31
100	106.249	106	4.76

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Stability Analysis:
The analytical data verify (see table) that the 1 and 100 mg/ml formulations were stable at room temperature for at least 8 days.

Nominal Value (mg/ml)	Content in %		Content as % of Nominal Value	
	Start	After 8 Days	Start	After 8 days
1	0.952	0.927	95	93
100	106.249	107.642	106	108

Concentration Analysis:
The analytical data verify that the test substance was homogeneously distributed in the 2 and 100 mg/ml formulations and that the contents were within defined limits.

Date of formulation preparation – May 22, 2002

Nominal Value (mg/ml)	Content (mg/ml)	Content as % of nominal value	%RSD
2	1.756	88	3.14
14	14.094	101	Content only
100	102.277	102	1.52

Date of formulation preparation – June 20, 2002

Nominal Value (mg/ml)	Content (mg/ml)	Content as % of nominal value	%RSD
2	1.822	91	11.82*
14	13.659	98	Content only
100	109.999	110	4.43

*Value not within defined limits

6. Dosage administration: The females were treated once daily by gavage between 06:00 and 12:30 a.m. from gestation days 6 through 19 post coitum. As in the preceding study with BYI 08330 (T9062786) the females were given the administration formulations orally by gavage.

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C. Observations

1. Maternal Observations and Evaluations: The animals were checked for mortality or clinical signs at least once daily. Body weight was recorded on gestation days 0 and daily between days 6 and 20. Food consumption was measured on gestation days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, and 18-20. Dams were sacrificed on day 20 of gestation. Examinations at sacrifice consisted of a gross pathological evaluation. Each uterus was weighed and dissected. The number of implantations was determined along with early resorptions, late resorptions, and dead fetuses. The number of corpora lutea in the ovaries was recorded as well.

2. Fetal Evaluations: The number of live fetuses was noted and their sex and body weight were recorded. All of the fetuses were evaluated for external abnormalities. Every other fetus was assigned to either the visceral or skeletal examinations. In the visceral examination, the fetus was sectioned according to the modified Wilson technique and the organs in the thoracic, abdominal and pelvic regions were evaluated for abnormalities. The fetuses included in the skeletal examination were eviscerated, treated with the cartilage staining technique of Inouye, using alcian blue GX, cleared with a dilute potassium hydroxide solution and stained with Alizarin red S.

D. Data analysis

1. Statistical analyses: The following parameters were analyzed using ANOVA with pairwise comparison for statistical significance by the Dunnett's test: food consumption, body weight, uterine weight, number of corpora lutea per female, number of implantations per female, number of live fetuses per female and as a percentage of implantations per female, placental weights per female and fetal weights per female. The Chi squared test was used to evaluate the number of fetuses or litters with visceral or cartilaginous tissue observations. The Chi square test was used to evaluate as well fertility and gestation rates, number of preimplantation losses per group, number of preimplantation losses, early resorptions, late resorptions or dead fetuses per group, the number of live fetuses per group in percent of implantations, number of male or female fetuses or fetuses with undeterminable sex per group, number of fetuses or litters with external, visceral or skeletal findings, and number of fetuses or litters with malformations. If a significant difference existed, Fisher's exact test with the Bonferroni correction was used for pairwise comparison. The Kruskal-Wallis test was used to evaluate the number of preimplantation losses per female, the number of postimplantation losses, early resorptions, late resorptions, or dead fetuses per female, number of male or female fetuses or fetuses of undeterminable sex per female, and the proportion of placental, fetal external or visceral findings per female. If a significant difference existed, Dunn's test was used for pairwise comparisons.

2. Historical control data: Historical control data were provided to allow comparison with concurrent study data. Control animal data from studies conducted between 1987 and 2003 were included in this database.

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II. Results

Findings:

Maternal toxicity: Mortality, clinical signs, feed and water intake as well as body weight development were not affected by treatment with BYI 08330.
Necropsy revealed no treatment related findings at a dose level up to and including 140 mg/kg bw/day. Absolute and relative liver weights were not affected by treatment with BYI 08330. Histopathology of the liver revealed no treatment-related findings.

Table 5.6.10-6: Mean body weights or body weight gains (g) of pregnant animals

Parameter	Dose Group (mg/kg bw/day)			
	0	10	35	140
Final body weight on day 20	**336.9**	**336.3**	**330.5**	**333.5**
Absolute body weight gain on day 6 - 19	**82.5**	**85.0**	**82.5**	**81.4**
Absolute body weight gain on day 0 - 20	**119.7**	**122.3**	**118.3**	**117.5**
Corrected body weight gain on day 0 – 20	**50.8**	**53.8**	**49.4**	**52.7**

Hematology /clinical chemistry: Red blood cell parameters and liver enzymes as well as substrates and electrolytes were not affected by treatment with BYI 08330.

Developmental toxicity: No effects occurred on gestation rate, postimplantation loss, number of fetuses, placental weight, placental appearance and fetal weights. Mean percentage of male fetuses was only 45.8% in the 140 mg/kg dose group of the actual study. However, a dose-response relationship was not evident in comparison to the preceding study with BYI 08330 at a dose level up to and including 1,000 mg/kg (Report No. AT01413), where the mean percentage of male fetuses per litter was 54.2 - 47.7 - 47.9 and 48.0 % in the control, 20 mg/kg, 140 mg/kg and 1,000 mg/kg dose groups, respectively. The mean of 45.8% includes one litter (female no. 1513) with one female fetus only. Without this litter mean percentage of males per litter is 47.9% in the 140 mg/kg group. Furthermore, both means lay in the range of historical control data (see Annex on page 699). Thus a treatment related effect on fetal sex distribution was not evident at a dose level up to and including 140 mg/kg bw/day.

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Table 5.6.10-7: Intrauterine development

Parameters	Dose Group (mg/kg bw/day)			
	0	10	35	140
Placental weight in g	**0.60**	**0.60**	**0.58**	**0.61**
Number of fetuses	**12.2**	**12.1**	**12.5**	**11.5**
Postimplantation loss	**0.5**	**1.1**	**0.6**	**0.5**
Males in %	**50.1**	**50.0**	**50.4**	**45.8**
Fetal weight in g	**3.60**	**3.61**	**3.57**	**3.60**

External, visceral and skeletal evaluation of fetuses revealed no treatment-related malformations at a dose level up to and including 140 mg/kg bw/day when excluding offsprings of male no. 16 from evaluation of this study. In the actual study all litters sired by this male had malformed fetuses with either missing head of 1st rib or microphthalmia or both. Male no. 16 was paired with one female of the control group (no. 1478), one female of the 10 mg/kg group (no. 1492), two females of the 35 mg/kg group (nos. 1503 and 1533; both had no implantation sites) and 4 females of the 140 mg/kg group.

Based on the high incidence and the specific type of malformations in the offsprings of male no. 16 in the actual and other contemporary developmental toxicity studies (see Annex of the study report on page 661 to 663 for a list of females of other studies paired with this male and the findings in their fetuses) it was appropriate to assume a paternally mediated effect for these malformations and it was regarded justified to exclude offsprings of male no. 16 from evaluation of this study. Though, only 19 litters were available at the 140 mg/kg dose level after exclusion of the 4 females mentioned above, however, the remaining numbers of fetuses and litters available for evaluation were still regarded adequate to fulfil the guideline requirements of approximately 20 litters per group.
The remaining malformations were generally of a common type and/or showed no dose response relationship on a fetal and/or litter basis either in comparison to the concurrent control or to the results of the preceding study.

Isolated malformations of the eyes, i.e. uni- and/or bilateral microphthalmia was the most common malformation in this study with a fetal incidence of 1.8% in the 35 mg/kg dose group and 0.4% in the 140 mg/kg dose group. Malformations of the eyes belong to the most common spontaneous malformations of the rat strain used (see historical control data on pages 950 to 953 in the Annex of this study report), were comparable with recent historical control data (up to 1.8% of fetuses and up to 20% of litters affected; cf. Annex on page 952) and displayed no dose relationship in the actual and in comparison to the preceding study. Thus the incidence of microphthalmias in this study was regarded as incidental and a treatment relationship was excluded.

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One lobe of the thyroid gland was absent in a fetus of the 35 mg/kg dose group and in 2 fetuses (in 2 litters) of the 140 mg/kg dose group. Treatment relationship was excluded for these findings due to lack of dose relationship in comparison to the preceding study with BYI 08330 with dose levels up to 1,000 mg/kg. In the former study the incidence of missing lobe of the thyroid gland comprised 3 fetuses in 2 litters of the control group and one fetus in the 140 mg/kg group while none of the fetuses of the 20 mg/kg and 1,000 mg/kg groups revealed this finding.
The remaining malformations in the treated groups, i.e. atrial septal defect of the heart, displaced azygos vein, right-sided aortic spur and dysplastic forelimb bones were single findings without dose response relationship and were comparable to the actual or historical controls so that treatment relationship was not evident for these findings.
In conclusion, the total number of malformed fetuses and litters revealed no dose response relationship in this study and the overall incidence lay well within the range of historical control data (see Annex of this report on pages 740 to 744).

Table 5.6.10-8: Summary of fetal malformations

Parameters	Dose Group (mg/kg bw/day)			
	0	10	35	140
Microphthalmia, unilateral and/or bilateral			5(5)	1
Lobe of thyroid gland absent			1	2(2)
Right sided aortic spur			1	
Right sided azygos vein			1	
Atrial septal defect of the heart	1	1		
4th thoracic vertebral body unilateral one ossification center missing and cartilage bipartite; bone and cartilage of 3rd and 5th thoracic vertebral bodies asymmetric	1			
4th and 5th thoracic vertebral bodies fused	1			
Dysplasia of forelimb bones (scapula, humerus, radius and/or ulna)	2(2)	1	1	
Supernumerary lumbar vertebra				1
Number of fetuses per group	266	263	275	227
Number of fetuses with malformations	5	2	9	3
Malformed fetuses per group (%)	1.9	0.8	3.3	1.3
Number of litters per group	22	22	22	19
Number of litters with malformations	5	2	9	3
Malformed litters per group (%)	22.7	9.1	40.9	15.8

( ) number of litters affected

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Fetal external and visceral examinations revealed no treatment-related deviations at a dose level up to and including 140 mg/kg bw/day.
Evaluation of fetal skeletal deviations showed considerably lower incidences of wavy ribs at all dose levels tested, when compared to the main study. Furthermore all means were comparable to the control group and thus revealed no indication for a treatment related effect. This also renders a treatment related effect for occurrence of wavy ribs at doses up to and including 140 mg/kg bw/day in the former study as well unlikely.

Table 5.6.10-9: Summary of fetal skeletal incidences

Parameter	Fetal and litter incidences (%) per Dose Group (mg/kg bw/day)							
	0		20		140		1,000	
Number of fetuses evaluated	**140**		**136**		**144**		**118**	
Wavy ribs (sum); fetal incidence	**9.3**		**8.8**		**13.2**		**9.3**	
14th ribs (sum); fetal incidence	**14.3**		**13.2**		**14.6**		**26.3**	
Number of litters evaluated	**22**		**22**		**22**		**19**	
Wavy ribs (sum); litter incidence	**36.4**		**22.7**		**27.3**		**21.1**	
14th ribs (sum); litter incidence	**54.5**		**40.9**		**36.4**		**47.4**	

Fetal skeletal examinations revealed statistically significantly retarded ossification of isolated bones on a fetal basis, scattered across all dose groups tested. However, a relationship to treatment was not assumed, based on the lack of reproducibility in almost all cases when compared with the preceding study. Moreover, none of the fetal means of the dose groups exceeded the limits of historical controls and no statistically significant changes have been observed on a litter basis. Furthermore, the dose-response correlation was generally poor.

The findings are described in detail as follows: Statistically significantly retarded ossification of digits of the forepaws was noted on a fetal basis (see table below). None of the findings at the forepaws was statistically significant on a litter basis, and the differences were poorly correlated with dose and lay in the range of historical control data at a dose level up to and including 140 mg/kg bw/day. Furthermore, the statistically significant findings at the 5th right digit showed no statistical significance in the preceding study at a dose level up to and including 140 mg/kg bw/day and the other localizations affected in the 140 mg/kg bw/day dose group in the actual study were not affected in the preceding study (except for distal phalanx of 4th digit left). Thus a treatment related effect on ossification of handbones was not assumed at a dose level up to and including 140 mg/kg bw/day in the actual study.

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Retarded ossification of distal phalanges of toes (incomplete and/or missing ossification) was seen with statistical significance on a fetal basis for distal phalanx of 3rd toe right in the 10 mg/kg dose group and of 3rd toe right and 2nd and 5th toe left at the 140 mg/kg bw/day dose level. On a litter basis statistical significance was not evident and all findings lay in the range of historical control data. In the preceding study with BYI 08330 the 3rd toe right and 5th toe left were not affected at a dose level up to and including 140 mg/kg bw/day. The 2nd toe left was as well affected in the preceding study with BYI 08330 but percentage of incompletely ossified 2nd toe left of the former study at 140 mg/kg bw/day (47.3%) was nearly identical to the control value of the actual study (45.3%). Thus, an effect on ossification of toes was not assumed at a dose level up to and including 140 mg/kg bw/day.

Incomplete ossification of 4th sacral vertebral arch left occurred in the 35 mg/kg and 140 mg/kg bw/day group with statistical significance on a fetal basis but without dose relation and within the range of historical control data. On a litter basis neither statistical significance nor dose relationship was seen for this finding. In the preceding study with BYI 08330 effects on ossification of sacral vertebrae was not evident at a dose level up to and including 140 mg/kg bw/day so that the findings were regarded as incidental and thus toxicologically relevant effects on ossification of sacral vertebral arches were not assumed at a dose level up to and including 140 mg/kg bw/day.

One bone of the skull (sphenoid bone) showed statistically significantly retarded ossification at the 140 mg/kg bw/day dose level on a fetal basis while ossification of the supraoccipital bones was more progressed (reduced number of incompletely ossified bones) at the 140 mg/kg bw/day dose level on a fetal and litter basis. On a litter basis neither statistical significance nor dose relationship were evident for retarded ossification of the sphenoid bone and the incidence lay in the range of historical control data on a fetal and litter basis. In the preceding study effects on ossification of supraoccipital and sphenoid bone were not observed at a dose level up to and including 140 mg/kg bw/day. Therefore a toxicologically relevant effect on ossification of skull bones was not assumed in the present study at a dose level up to and including 140 mg/kg bw/day.

Statistically significantly increased numbers of punctiform and comma-shaped supernumerary ribs (14th ribs) were observed in the 140 mg/kg bw/day dose group. However, statistical significance was not observed for percentage of overall number of 14th ribs on a fetal basis nor for the incidence of 14th ribs on a litter basis. The highest overall percentage of 14th ribs on litter basis even occurred in the control group (54.5% of litters affected versus 47.4% in the 140 mg/kg bw/day group). Furthermore, a treatment related effect on incidence of 14th ribs was not evident in the preceding study with BYI 08330 at a dose level up to and including 140 mg/kg bw/day. Therefore a treatment related influence on the incidence of supernumerary ribs was not assumed at a dose level up to and including 140 mg/kg bw/day.

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Table 5.6.10-10: Summary of fetal skeletal deviations

Parameter	NR	Dose Group (mg/kg bw/day)				NR
		0	**10**	**35**	**140**	
2nd digit distal phalanx -r: incompl ossif.	**74.4**	51.4	61.0	53.1	69.5 *	**25.7**
4th digit distal phalanx -r: incompl ossif.	**66.9**	37.9	46.3	45.8	59.3 **	**13.7**
5th digit distal phalanx -r: incompl ossif.	**96.3**	83.6	94.1 *	93.1 *	95.8 **	**63.2**
2nd digit distal phalanx -l: incompl ossif.	**79.8**	60.7	70.6	60.4	75.4 *	**32.7**
3rd digit distal phalanx -l: incompl ossif.	**45.7**	24.3	27.9	31.3	42.4 **	**8.8**
4th digit distal phalanx -l: incompl ossif.	**74.8**	45.7	59.6	48.6	63.6 *	**22.4**
4th digit proximal phalanx -r: incompl ossif.	**8.3**	25.7	16.2	16.7	12.7 *	**31.9**
3rd digit proximal phalanx -l: incompl ossif.	**9.3**	25.0	14.7	11.8 *	15.3	**23.8**
4th digit proximal phalanx -r: unossified	**91.0**	71.4	83.3	82.6	87.3 *	**65.6**
3rd digit proximal phalanx -l: unossified	**88.9**	75.0	85.3	87.5 *	84.7	**66.4**
3rd toe distal phalanx -r: incompl ossificat.	**47.2**	25.4	40.7 *	30.8	44.1 **	**15.9**
2nd toe distal phalanx -l: incompl ossificat.	**62.0**	45.3	59.6	52.1	61.9 *	**23.0**
5th toe distal phalanx -l: unossified.	**3.4**	7.2	9.6	11.8	18.6 *	**30.0**
5th sternal segment: incompl ossification	**55.6**	12.9	27.2 *	17.4	19.5	**6.8**
Sacral vertebral arch 4 -: incomplete ossif.	**44.1**	12.9	24.3	26.4 **	25.4 *	**15.0**
Sphenoid bone: incomplete ossification	**9.0**	0.7	5.9	2.8	7.6 *	**0.0**
Supraoccipital bone: incomplete ossification	**15.1**	11.4	8.1	13.2	0.8 **	**1.8**

NR = normal range limit; * = $p < 0.05$; ** = $p < 0.01$

III. Conclusion: The NOAEL for maternal toxicity was 140 mg/kg bw/day, based on the absence of effects at 140 mg/kg bw/day. The NOAEL for developmental toxicity was established at 140 mg/kg bw/day, based on the absence of treatment-related deviations at 140 mg/kg bw/day. No evidence for a primary embryotoxic or teratogenic potential of BYI 08330 was determined.

Based on the results of the supplementary study with BYI 08330 no indication for a treatment related increase of wavy ribs and no evidence for retarded ossification was noted at a dose level up to and including 140 mg/kg bw/day.

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IIA 5.6.11 Teratogenicity test by the oral route in the rabbit

Report:	**KIIA 5.6.11/01, [redacted] & [redacted]; 2001**
Title:	BYI 08330 - Pilot developmental toxicity study in rabbits after oral administration
Report No & Document No	T3062735 M-084392-01-2
Guidelines:	Not required. Deviations: none
OECD/FIFRA GLPS	No

Executive Summary

In the present pilot developmental toxicity study, groups of 3 inseminated female Himalayan rabbits each were treated daily, orally by gavage, with BYI 08330, batch no. NLL 6425-7, purity: 97.0%, suspended in 0.5% aqueous carboxymethylcellulose, from day 6 to day 28 post coitum (p.c.) at doses of 0, 5, 25, 100, 160, 250 or 500 mg/kg bw/day. The fetuses were delivered by cesarean section on day 29 of gestation. Investigations were performed on general tolerance of the test compound by the females as well as on its effect on intrauterine development (including external and visceral evaluation of fetuses).

Maternal effects were noted already at the 160 mg/kg dose level (body weight loss, impaired body weight development, decreased feed intake) and was severe (hypoactivity, labored breathing, convulsion, severe body weight loss, severely decreased feed intake) at levels of 250 mg/kg and above resulting in death or premature sacrifice in moribund conditions in all animals treated at 500 mg/kg bw/day and in one animal of the 250 mg/kg dose level. Necropsy revealed in single animals hardened abdominal fatty tissue, round depressions in the gastric mucosa, enlarged gall bladder, or gaseous content in the intestine and hemorrhages in the renal capsule.

The gestation rate in the 250 mg/kg group was decreased by one abortion and by one total resorption. Due to these findings and due to early sacrifice or death of the remaining females in the 250 and 500 mg/kg groups the evaluation of the remaining reproduction parameters was limited to dose levels up to and including 160 mg/kg. Effects on intrauterine development could not be completely excluded at the 160 mg/kg level (possibly marginally reduced fetal and placental weights).

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Results and Discussion:

Maternal toxicity: One female of the 500 mg/kg group died on day 10 p.c. The remaining two females of this group and one female of the 250 mg/kg group were killed for human reasons on day 10 or 18 p.c. (500 mg/kg) or day 23 p.c. (250 mg/kg). One further female of the 250 mg/kg group was killed after abortion on day 24 p.c. Two females of the 500 mg/kg group and the female of the 250 mg/kg group, which was killed in moribund conditions, revealed wounds in the region of the head, throat or forelimbs. Hypoactivity (lying on side) on a single day occurred in one female each of the 500 and 250 mg/kg groups as well as in the female with abortion in the 250 mg/kg group which also showed labored breathing and convulsion. Cold ears, severely decreased or no feed intakes and distinct to severe body weight loss (up to - 343 g) also occurred in the females of the 500 and 250 mg/kg groups. Necropsy revealed hardened fatty tissue in the abdominal cavity and at the border of the pancreas and round depressions in the gastric mucosa in one female of the 500 mg/kg group, an enlarged gall bladder in one female and gaseous contents in the stomach and intestine as well as hemorrhages in the renal capsule in a further female of the 250 mg/kg group.

Cold ears occurred for several days in all females of the 160 mg/kg group. Feed intakes were transiently distinctly to severely decreased in two females at the 160 mg/kg level, which also showed moderate to distinct body weight loss (-191 or -273g). Gross necropsy did not reveal treatment related findings at levels up to and including 160 mg/kg.

Developmental toxicity: The gestation rate in the 250 mg/kg group was decreased by one abortion and by one total resorption. Due to this abortion, total resorption, early sacrifice or death of all females in the 250 and 500 mg/kg groups the evaluation of the remaining reproduction parameters was limited to dose levels up to and including 160 mg/kg.

The resorption rate and correspondingly the number of fetuses as well as fetal sex distribution were unaffected at levels up to and including 160 mg/kg. Placental and fetal weights in the 160 mg/kg group were slightly decreased when compared to the control group which was, however, most likely due to the incidentally higher litter sizes in the 160 mg/kg group rather than a treatment-related effect. A final assessment is, however, not possible due to the low number of females in a pilot study. The isolated malformations seen in one fetus each of the 100 mg/kg group (malposition of forelimb) and in the 25 and 160 mg/kg groups (cardiac ventricular septal defect with/without truncus arteriosus) are considered incidental as they are known as common findings in the strain of rabbits used.

Conclusion: Based on these results, it is concluded that the dose level of 160 mg/kg bw/day is suitable for the high dose level in the main developmental study of BYI 08330 in rabbits. It is also considered that the dose levels of approximately 40 mg/kg bw/day and 10 mg/kg bw/day are suitable for the middle and low dose levels, respectively.

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Report:	**KIIA 5.6.11/02, ; 2001**
Title:	BYI 08330 - Developmental toxicity study in rabbits after oral administration
Report No &	AT01003
Document No	M-122324-01-2
Guidelines:	OECD 414 (2001), 88/302/EEC (1988), US-EPA-OPPTS 870.3700 (1998), US EPA 712-C-98-207, JMAFF 12-Nousan-No. 8147 (2000, amended 2001); Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In the present developmental toxicity study, groups of 22, 22, 24 or 30 female Himalayan rabbits were orally treated (gavage) from day 6 to 28 post coitum (p.c.) with BYI 08330 (batch no. 08045/0004, purity: 98.9 %) at doses of 0, 10, 40 or 160 mg/kg bw/day, respectively. The test compound was formulated as a suspension with 0.5 % carboxymethylcellulose in demineralised water. On the 29th day of gestation the fetuses were delivered by cesarean section. Doses were selected based on the results of a pilot study in which doses of 0, 5, 25, 100, 160, 250 or 500 mg/kg bw/day BYI 08330 were tested. The results were documented in a separate study (Report No. T3062735). The stability and homogeneity of 1.0 and 100mg/mL formulations, and the achieved concentrations of all formulations, were confirmed by analysis during the study.

One female of the 160 mg/kg group was found dead and 5 females were sacrificed in moribund condition with severely reduced feed intake, severe body weight loss, cold ears, alopecia, reduced amount of feces, diarrhea or soft and light colored feces, reddish excretion, decreased water intake and urination and discolored urination. Females at necropsy revealed fluid and/or gaseous contents in caecum, discolored liver and mottled gall bladder. Abortion was observed most likely as a consequence of maternal toxicity in two other females of the 160 mg/kg group and in one female of the 40 mg/kg group showing clinical symptoms, impaired feed intake and body weight loss before abortion. The remaining females of the 160 mg/kg dose group revealed cold ears, alopecia and soft, mucoid and light colored feces.

The gestation rate was decreased by one abortion in the 40 mg/kg dose group for which a treatment related effect cannot be excluded and two abortions in the 160 mg/kg group most likely as a consequence of severe maternal toxicity. There were no treatment-related effects in the remaining females on postimplantation loss, on the number of fetuses as well as on placental weight and appearance; fetal weight and fetal sex distribution were unaffected by treatment at dose levels up to and including 160 mg/kg bw/day.

Teratogenic effects of BYI 08330 were excluded at a dose level up to and including 160 mg/kg bw/day. Malformations were different in type, scattered within the different dose groups and revealed no dose dependency. Malformations of cartilaginous parts of ribs, cardiac ventricular septal defects and malpositioned forelimbs were not dose related and single malformations which appeared only once in the 160 mg/kg dose group (domed head together with encephalomeningocele, cleft palate, microphthalmia) were different in type and comparable with historical control data.

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Besides possibly increased incidence of fetuses with distinct liver lobulation in the 160 mg/kg group, a treatment related effect on fetal external and visceral deviations was not assumed at dose levels up to and including 160 mg/kg. Fetal skeletal - including cartilaginous tissue – evaluation revealed no treatment related effects at a dose level up to and including 160 mg/kg bw/day.

Findings on the progeny (abortions at the 40 mg/kg and 160 mg/kg dose level; possibly distinct fetal liver lobulation at the 160 mg/kg dose level) occurred only at dose levels with signs of distinct to severe maternal toxicity.

The following NOAELs were thus determined in this developmental toxicity study:

Systemic maternal toxicity: 10 mg/kg body weight/day

Abortions: 10 mg/kg body weight/day

Fetuses: 40 mg/kg body weight/day

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330

Description:	technical, light-beige powder, room temp storage
Lot/Batch No.:	08045 / 0004
Purity (ai%):	98.9 %
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No.of TGAI:	203313-25-1

2. Vehicle and/or positive control: 0.5% carboxymethylcellulose (CMC high viscosity, by Fluka █ █, Switzerland) in demineralized water as a suspension (vehicle)

3. Test animals:

Species:	Rabbit, Himalayan	
Strain:	CHBB:HM	
Age/weight at study initiation:	106 to 192 days old 1929 to 2805g (day 0 post coitum)	
Source:	█, Germany	
Housing:	Individually housed in Makrolon cages	
Diet:	█ rabbit diet K- Z *ad libitum* (█ █, Germany)	
Water:	Tap water *ad libitum*	
Environmental conditions:	**Temperature:**	20 ± 2ºC
	Humidity:	50 ± 20%
	Air changes:	at least 10/hr
	Photoperiod:	12 hr light / dark cycle
Acclimatisation:	At least 7 days	

B. Procedures and study design

1. In life dates: Start: March 25, 2003; End: June 25, 2003

2. Mating: The mating was performed between 05:00 and 10:00 C E T. One male rabbit was mated with one female rabbit under observation. About one hour after the first mating had occurred the same animals were mated again. It was recorded which female was mated with which male. The day on which the copulation was observed was considered as day 0 of gestation.

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3. Animal Assignment*: After copulation, 88 females were sequentially allocated to 4 experimental groups according to a computer-generated randomization procedure. The study groups are indicated in Table 1.

Dose (mg/kg bw/day)	0	10	40*	160*
Females	22	22	22	22

*During the conduct of the study, 10 further females were allocated to the study without randomization: 2 females to the 40 mg/kg dose group and 8 females to the 160 mg/kg dose group.

4. Dose selection rationale: The dose levels used were selected according to a dose-range finding-study in rats with dose levels of 0, 50, 200, 800 and 1,000 mg/kg (Report No. T3068559). Maternal effects were seen at the 800 mg/kg dose level (body weight loss, impaired body weight development) and distinct at the 1,000 mg/kg dose level (respiratory findings, piloerection, body weight loss, impaired body weight gain, in- or decreased water intake, increased urination, light colored feces). Effects on intrauterine development could not be completely excluded at the 200 rng/kg level (possibly marginally reduced fetal weight), were evident at a dose level of 800 mg/kg (reduced placental and possibly fetal weight, retarded ossification, wavy ribs) and were clearly observed at the 1,000 mg/kg dose level (distinctly reduced fetal and placental weight, necrotic placental borders, retarded ossification, wavy and 14th ribs and possibly marginally increased incidence of common malformations).

5. Dosage preparation and analysis: Prior to the start of the study, stability of the test substance in aqueous 0.5% carboxymethyl-cellulose was evaluated for a period of 8 days at room temperature. Concentration and homogeneity (top, middle, and bottom) of the test mixture were evaluated once prior to the study and once during the study. Investigations on the stability and homogeneity and a content check of the active ingredient in samples of 1 mg/ml and 100 mg/ml were performed before the start of this study and covered the concentrations used in this study. The first content check of the formulations of all concentrations as well as checks on homogeneity of the low and high concentrations revealed no significant deviation of the active ingredient content from the nominal value in the formulations of any of the treatment groups and homogeneity of the low and high concentrations also complied.

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Results:

Homogeneity Analysis:

The analytical data verify (see table) that the test substance was homogeneously distributed in the 1 and 100 mg/ml formulation (Suspensions had to be stirred to achieve homogenization)

Nominal Value (mg/ml)	Content (mg/ml)	Content as % of nominal value	%RSD
1	0.952	95	0.31
100	106.249	106	4.76

Stability Analysis:

The analytical data verify (see table) that the 1 and 100 mg/ml formulations were stable at room temperature for at least 8 days.

Nominal Value (mg/ml)	Content in %		Content as % of Nominal Value	
	Start	After 8 Days	Start	After 8 days
1	0.952	0.927	95	93
100	106.249	107.642	106	108

Concentration Analysis:

The analytical data verify that the test substance was homogeneously distributed in the 2 and 100 mg/ml formulations and that the contents were within defined limits.

Date of formulation preparation – May 22, 2002

Nominal Value (mg/ml)	Content (mg/ml)	Content as % of nominal value	%RSD
2	1.756	88	3.14
14	14.094	101	Content only
100	102.277	102	1.52

Date of formulation preparation – June 20, 2002

Nominal Value (mg/ml)	Content (mg/ml)	Content as % of nominal value	%RSD
2	1.822	91	11.82*
14	13.659	98	Content only
100	109.999	110	4.43

*Value not within defined limits

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6. Dosage administration: The females were treated once daily by gavage between 06:00 and 12:30 a.m. from gestation days 6 through 19 post coitum. The females of the control group received vehicle only (0.5% aqueous CMC) at the same volume. Dosing was based on the body weight from the most recent body weight determination, using a uniform dose volume of 10 ml/kg body weight.

C. Observations

1. Maternal Observations and Evaluations: The animals were checked for mortality or clinical signs at least once daily. Body weight was recorded on gestation days 0 and daily between days 6 and 20. Food consumption was measured on gestation days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, and 18-20. Dams were sacrificed on day 20 of gestation. Examinations at sacrifice consisted of a gross pathological evaluation. Each uterus was weighed and dissected. The number of implantations was determined along with early resorptions, late resorptions, and dead fetuses. The number of corpora lutea in the ovaries was recorded as well.

2. Fetal Evaluations: The number of live fetuses was noted and their sex and body weight were recorded. All of the fetuses were evaluated for external abnormalities. Every other fetus was assigned to either the visceral or skeletal examinations. In the visceral examination, the fetus was sectioned according to the modified Wilson technique and the organs in the thoracic, abdominal and pelvic regions were evaluated for abnormalities. The fetuses included in the skeletal examination were eviscerated, treated with the cartilage staining technique of Inouye, using alcian blue GX, cleared with a dilute potassium hydroxide solution and stained with Alizarin red S.

D. Data analysis

1. Statistical analyses: The following parameters were analyzed using ANOVA with pairwise comparison for statistical significance by the Dunnett's test: food consumption, body weight, uterine weight, number of corpora lutea per female, number of implantations per female, number of live fetuses per female and as a percentage of implantations per female, placental weights per female and fetal weights per female. The Chi squared test was used to evaluate the number of fetuses or litters with visceral or cartilaginous tissue observations. The Chi square test was used to evaluate as well fertility and gestation rates, number of preimplantation losses per group, number of preimplantation losses, early resorptions, late resorptions or dead fetuses per group, the number of live fetuses per group in percent of implantations, number of male or female fetuses or fetuses with undeterminable sex per group, number of fetuses or litters with external, visceral or skeletal findings, and number of fetuses or litters with malformations. If a significant difference existed, Fisher's exact test with the Bonferroni correction was used for pairwise comparison. The Kruskal-Wallis test was used to evaluate the number of preimplantation losses per female, the number of postimplantation losses, early resorptions, late resorptions, or dead fetuses per female, number of male or female fetuses or fetuses of undeterminable sex per female, and the proportion of placental, fetal external or visceral findings per female. If a significant difference existed, Dunn's test was used for pairwise comparisons.

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2. Historical control data: Historical control data were provided to allow comparison with concurrent study data. Control animal data from studies conducted between 1987 and 2003 were included in this database.

II. Results and Discussion:

Maternal toxicity: One female of the 160 mg/kg group was found dead after having shown reddish excretion and soft feces and 5 females had to be sacrificed in moribund condition after having shown severely reduced to no feed intake, severe body weight loss, cold ears, alopecia, reduced amount or no feces, diarrhea (in one female) and soft and light colored feces, reddish excretion, decreased water intake and urination and discolored urination most probably indicating concentration of urine. Females which had to be sacrificed in moribund condition revealed fluid and/or gaseous contents in caecum, discolored liver and mottled gall bladder at necropsy. Abortion was observed most likely as a consequence of maternal toxicity in two other females of the 160 mg/kg group and in one female of the 40 mg/kg group showing clinical symptoms, impaired feed intake and body weight loss before abortion. The remaining females of the 160 mg/kg dose group revealed cold ears, alopecia and soft, mucoid and light colored feces.
A statistically significantly reduced feed intake was noted in the 10 mg/kg dose group (day 27-29 p.c.). This finding was regarded as incidental because it was not dose related and was within the range of historical control data so that the finding was regarded as unrelated to treatment.
Statistically significantly reduced body weight gain during treatment (days 6-29 p.c.) and the overall gestation period (days 0-29 p.c.) was noted in the 10 mg/kg dose group which resulted in a marginal body weight loss when corrected body weight gain was taken into consideration. Impaired body weight development at the 10 mg/kg dose level was regarded as an incidental finding and without relation to treatment due to lack of dose dependency. Mortality or early sacrifice at the 40 mg/kg and 160 mg/kg dose levels did not contradict the assumed lack of dose dependency since only one female was sacrificed due to abortion in the 40 mg/kg dose group and a negative impact of mortality on the evaluation of body weight development could thus be excluded at a dose level up to and including 40 mg/kg.

Table 5.6.11-1: Mean body weights or body weight gains (g) of pregnant animals

Parameter		Dose Group (mg/kg bw/day)							
		0		10		40		160	
Final body weight	**day 29**	**2,735.5**		**2,751.1**		**2,815.8**		**2,738.1**	
Absolute body weight gain	**day 6 - 29**	**362.0**		**287.9**	*	**355.3**		**327.3**	
Absolute body weight gain	**day 0 - 29**	**473.5**		**376.9**	**	**477.0**		**418.2**	
Corrected body weight gain	**day 0 – 29**	**65.2**		**-0.8**		**60.8**		**46.5**	

* = p < 0.05; ** = p < 0.01

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Developmental toxicity: The gestation rate was decreased by one abortion in the 40 mg/kg dose group for which a treatment related effect cannot be excluded and two abortions in the 160 mg/kg group most likely as a consequence of severe maternal toxicity. Postimplantation loss in the remaining females, the number of fetuses as well as placental and fetal weight and appearance, and fetal sex distribution were unaffected by treatment at dose levels up to and including 160 mg/kg bw/day.

Table 5.6.11-2: Intrauterine development

Parameters	Dose Group (mg/kg bw/day)			
	0	10	40	160
No. of mated females	**22**	**22**	**24**	**30[a]**
No. of mated females evaluated	**22**	**22**	**24**	**24**
No. of females with implantations	**22**	**22**	**22**	**21**
- in % of those mated	**100**	**100**	**91.7**	**87.5**
Females with viable fetuses	**22**	**22**	**21**	**19**
- in % of those with implantat.	**100**	**100**	**95.5**	**90.5**
Females with abortions	**0**	**0**	**1**	**2**
Mean number of fetuses/litter[b]	**7.7**	**6.9**	**8.2**	**6.9**
Postimplantation loss	**0.5**	**0.6**	**0.4**	**0.3**
Males in % [b]	**49.5**	**47.2**	**46.0**	**51.4**
Fetal weight in g [b]	**37.78**	**38.11**	**36.25**	**38.48**

[a]6 females excluded, which died or were sacrificed in moribund condition; [b]without females with abortions

Malformations were different in type (one fetus each with domed head/encephalomeningocele, cleft palate, microphthalmia or supernumerary presacral vertebra and several fetuses with malposition of forelimbs, malformations in the cartilaginous parts of ribs/cervical ribs/supernumerary ossification centers or ventricular septal defects of the heart), scattered within the different dose groups and revealed the highest total incidence of malformations in the 10 mg/kg group. The total incidence of malformed fetuses and litters in all dose groups lay well within the normal range of scattering for the strain of rabbits used and showed no dose dependency.

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Malformations in the cartilaginous parts of ribs, of the heart and malpositioned forelimbs (one of the most common malformations in the rabbit strain used and most likely due to restriction of fetal movement in the uterus) were not dose related and/or lay well within the range of normal scattering of the rabbit strain used and/or were as well seen in the current control. Furthermore, single malformations which appeared only once in the 160 mg/kg dose group (domed head together with encephalomeningocele, cleft palate, microphthalmia) were different in type and comparable with historical control data so that a treatment related effect was not assumed for these malformations. Thus, a teratogenic effect of BYI 08330 was excluded at a dose level up to and including 160 mg/kg bw/day. As some fetuses of the 10 mg/kg and 160 mg/kg group revealed more than one malformation, the number of malformations is higher than the number of affected fetuses in these groups.

Table 5.6.11-3: Summary of fetal malformations

Parameters	**Dose group (mg/kg bw/day)**			
	0	**10**	**40**	**160**
Eyeball reduced in size (microphthalmia)				**1**
Domed head/encephalomeningocele				**1**
Cleft palate				**1**
Malpositioned forelimbs	**1**	**3(2)**	**1**	**5(2)**
Ventricular septal defect of the heart	**1**	**3(3)**		
Fusion, bifurcation in the cartilaginous part, or slight thickening of ribs with/without supernumerary ossification center above the 1st	**1**	**3(3)**	**1**	**1**
sternal segment or at 8th rib cervical rib, supernumerary ossification center (with cartilage) above the 1st sternal segment fused with it			**1**	
Missing presacral vertebra	**2(2)**			
Supernumerary presacral vertebra			**1**	
Number of fetuses per group	**170**	**152**	**172**	**132**
Number of fetuses with malformations	**5**	**8**	**4**	**7**
Malformed fetuses per group (%)	**2.9**	**5.3**	**2.3**	**5.3**
Number of litters per group	**22**	**22**	**21**	**19**
Number of litters with malformations	**5**	**7**	**4**	**4**
Malformed litters per group (%)	**22.7**	**31.8**	**19.0**	**21.1**

( ) number of litters affected

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Fetal external and visceral evaluations revealed a possibly increased incidence of fetuses with distinct liver lobulation in the 160 mg/kg group.
Few fetuses revealed a slight dilation of lateral brain ventricles and whitish discoloration of the liver: The findings were comparable either to the current controls(slight dilation of brain ventricles) or to historical controls (up to 2.8% of fetuses and 14.3% of litters with whitish liver discoloration) and revealed no dose dependency.

Table 5.6.11-4: Summary of fetal malformations

Parameters	Dose group (mg/kg bw/day)			
	0	10	40	160
Slight dilation of lateral brain ventricles	5(2)	2(2)	3(2)	
Whitish discoloration of the liver		1	3(2)	2(1)
Distinct liver lobulation			2(1)	8**(2)
Number of fetuses per group	170	152	172	132
Number of fetuses with deviations	5	3	8	10*
Fetuses with deviations per group (%)	2.9	2.0	4.7	7.6
Number of litters per group	22	22	21	19
Number of litters with deviations	2	3	5	3
Litters with deviations per group (%)	9.1	13.6	23.8	15.8

( ) number of litters affected, * = $p < 0.05$, ** = $p < 0.01$

Fetal skeletal evaluations (retardations, variations) including cartilagineous tissues revealed no treatment-related effects at a dose level up to and including 160 mg/kg bw/day. Single statistically significant differences were observed at the 10 mg/kg and 40 mg/kg levels when calculation was done on a fetal basis (retarded ossification of the 5th sternebra in the 10 mg/kg dose group and of the 8th caudal vertebral arches in the 10 mg/kg and 40 mg/kg group). All values regarding retarded ossification at the 10 mg/kg and 40 mg/kg levels lay well within the normal range of scattering for these findings in developmental toxicity studies in the strain of rabbits used. A dose dependency was not evident for these findings and statistical significance was not seen when calculation was done on a litter basis.

III. Conclusion: The NOAEL for maternal toxicity was 10 mg/kg bw/day, based on abortion in one animal at 40 mg/kg bw/day with clinical symptoms, impaired feed intake and body weight loss before abortion. The NOAEL for abortion was 10 mg/kg bw/day, based on abortion in one animal at 40 mg/kg bw/day, most likely secondary to severe systemic maternal toxicity. The NOAEL for embryo/fetal development was 40 mg/kg bw/day, based on a possibly increased incidence of fetuses with distinct liver lobulation at 160 mg/kg bw/day. There was no evidence for an embryotoxic or a teratogenic potential.

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IIA 5.7 Neurotoxicity

Based on specific neurotoxicity screening assessments conducted acutely in the rat, BYI 08330 did not provide any qualitative or quantitative evidence of a neurotoxic potential.
Evidence of toxicity was limited to clinical signs (urine and perianal stain) and decreased activity in the figure-eight maze, beginning on the day of treatment with complete recovery by day 7. There were no compound-related gross or microscopic lesions up to and including the limit dose of 2000 mg/kg.

IIA 5.7.1 Acute neurotoxicity – rat

Report:	**KIIA 5.7.1/01, [redacted], 2005**
Title:	An acute oral neurotoxicity screening study with technical grade BYI 08330 in Wistar rats
Report No &	201283
Document No	M-254187-01-1
Guidelines:	US EPA OPPTS 870.6200, OECD 424, Health Canada PMRA DACO No. 4.5.12. Deviations: none
OECD/FIFRA GLPS	Yes. Deviations: none

Executive Summary

In the present acute neurotoxicity study BYI 08330, mix batch no. 08045/0014, purity 97.8 %–98.5 %, was administered by gavage in a single dose to nonfasted young-adult Wistar rats (12/sex/dose level), using nominal doses of 0 (vehicle), 200, 500 and 2,000 mg/kg for males and females. Since there were compound-related effects at 200 mg/kg, a follow-up study was conducted under the same conditions at nominal doses of 0 (vehicle), 50, 100 and 500 mg/kg in males and females to verify the findings at 500 mg/kg and to establish an overall NOAEL.
The test substance was suspended in 0.5% methylcellulose / 0.4% Tween 80 in deionized water and administered at a dosing volume of 10 ml/kg. Treatment-related observations involved urine stain in males and females at 200 mg/kg and above and perianal stain in males at 500 mg/kg and above. Locomotor activity (LMA) was reduced in males at 200 mg/kg and above and in females at 500 mg/kg and above. Interval MA and LMA were reduced at 500 mg/kg at several intervals in males and – to a lesser extent - in females. A slight reduction in interval LMA was still observed in males at 200 mg/kg. No treatment-related effects were observed at 50 and 100 mg/kg in both sexes.

The LOAEL for this study is 182 mg/kg bw/day. In males the LOAEL is based on urine stain (clinical observations and FOB) and decreased locomotor activity. In females the LOAEL is based on urine stain (clinical observations). The NOAEL is 100 mg/kg bw/day.

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I. Material and methods

A. Materials:

1. Test Material:	BYI 08330
Description:	Technical grade, white powder, room temp storage
Lot/Batch No.:	Mix batch 08045 / 0014
Purity (a.i. %):	97.8 % (12-2003); 98.5 (12-2005)
Chemical Name:	3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
CAS No. of TGAI:	*trans*: 382608-10-8; *cis*: 203313-25-1

2. Vehicle and/or positive control: The vehicle used was 0.5% methylcellulose/0.4% Tween 80 in deionized water

3. Test animals:

Species:	Rat; male and female [nulliparous and non-pregnant]	
Strain:	Wistar Hanover (Crl:WI[Glx/BRL/Han]IGS BR)	
Age/weight at dosing:	~9 weeks / males: 238-304 g; females: 155-164 g	
Source:	[redacted]	
Housing:	Animals were individually housed	
Diet:	[redacted] Chow 5002 was provided *ad libitum*	
Water:	Tap water was provided *ad libitum*	
Environmental conditions:	**Temperature:**	18-26°C
	Humidity:	30-70%
	Air changes:	10 air changes/hr minimum
	Photoperiod:	12 hrs light / 12 hrs dark
Acclimatisation:	At least 6 days	

B. Study design:

1. In life dates: Start: 4/5/2004 End: 11/25/2004

2. Animal assignment and treatment: The animals were weighed and those with body weights that were more or less than 20% of the mean weight for each sex were rejected. Using software, the remaining animals were randomly assigned to the control or a treatment group so that, for each sex, groups had comparable body weights when treatment was started. Fasted rats were administered a single dose by gavage in 0.5% methylcellulose/0.4% Tween 80 in deionized water at a dosing volume of 10 ml/kg then observed at least once daily for mortality or clinical signs of moribundity and weighed (as part of the FOB) one week prior to treatment, approximately 4 hours after treatment, and 7 and 14 days after treatment.

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The rationale for dose selection was based on the results of an acute oral toxicity (LD50) study in young adult female Wistar rats. In that study, fasted female Wistar rats were administered an acute oral (gavage) dose of 2,000 mg/kg as an aqueous suspension in 0.5% carboxymethylcellulose, at a dosing volume 10 ml/kg. Animals were observed for mortality and clinical signs for 14 days after treatment. The test substance produced no clinical signs or mortality at the limit dose. These results support the use of a limit dose (2,000 mg/kg) in the neurotoxicity study but provide no information to establish the time of peak effect. Thus, the results from a study using radio-labeled BYI 08330 were examined to estimate the time of peak effect. In that study, adult Wistar rats received a single oral (gavage) dose of 100 mg/kg (both sexes) or 1,000 mg/kg (males only) BYI 08330 in 0.5% (w/v) tragacanth in water adjusted to pH 4 with acetic acid, at a dosing volume of 10 ml/kg. In this study, the group mean peak blood concentrations occurred 1.0 to 3.0 hours (males) or 0.67 to 1.0 hour (females) after treatment at 100 mg/kg and 1.0 to 8.0 hours (males only) following administration at 1,000 mg/kg. Plasma concentrations in males at a dose of 1,000 mg/kg (2% cremophor in water) changed very little at 1.0, 2.0, 4.0 and 8.0 hours following treatment (570, 600, 770 and 700 µM, respectively). Based on these collective results, the doses selected for the initial study were 0, 200, 500 and 2,000 mg/kg for both sexes. The 2,000 mg/kg dose was selected as a limit dose that may produce slight evidence of toxicity, the middle dose was selected to produce minimal or no effect and the low dose was expected to be an overall NOEL in both sexes. Based on the initial findings, it was necessary to conduct a follow-up study to establish a NOAEL for clinical observations, FOB and measures of activity. In that study, doses selected were 0, 50, 100 and 500 mg/kg. The 500 mg/kg dose was selected to reproduce the findings in the initial study. The remaining treated doses were selected to establish an overall NOAEL. Based on the estimated time of peak blood concentrations at these dose ranges, the FOB began approximately 90 minutes (minimum) following dose administration, with the automated test of activity concluding at about 4 hours after treatment. Animals were assigned to the test groups noted in the following table.

Experimental Parameter	Dose Group (mg/kg bw/day)					
	0	50	100	200	500	2,000
Total No. Animals/sex/grp	12/sex	12/sex	12/sex	12/sex	12/sex	12/sex
Behavioral Testing	12/sex	12/sex	12/sex	12/sex	12/sex	12/sex
Neuropathology	6/sex	6/sex	6/sex	6/sex	6/sex	6/sex

n=12 animals/dose group for both studies

3. Test Substance Preparation and Analysis: The test substance was suspended in 0.5% methylcellulose/0.4% Tween 80 in deionized water. No information on how the test substance was stored was provided. No information on how stability, concentration, and homogeneity were verified was provided.

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Results

Homogeneity Analysis:

Homogeneity of the test substance in the vehicle was accepted for the range of concentrations used here, as the 4, 15 and 200 mg/ml concentrations had percent relative standard deviations (%RSD) of 0.90%, 2.1% and 2.5%, respectively.

Stability Analysis:

No appreciable decrease in concentration with eight days of storage for nominal concentrations of 4, 15 or 200 mg/ml (equivalent to doses of 40, 150 or 2,000 mg/kg, respectively).

Concentration Analysis:

For the **initial** study, doses of 0, 200, 500 and 2,000 mg/kg for males and females ranged from 91% to 103% of the nominal concentrations. Based on these results, the analytically-confirmed doses for males and females were 0, 182, 515 and 1930 mg/kg. For the **follow-up** study, doses of 0, 50, 100 and 500 mg/kg for males and females ranged from 95% to 105% of the nominal concentrations. Based on these results, the analytically-confirmed doses for males and females were 0, 47.4, 99.8, 523 mg/kg.

Statistics:

Generally, continuous data were analyzed using ANOVA, followed by Dunnett's test. For FOB, continuous data were first analyzed using Repeated-Measures ANOVA followed by a one-way ANOVA followed by Dunnett's test and categorical data were analyzed using General Linear Modeling and Categorical Modeling with post-hoc comparisons using Dunnett's test and an Analysis of Contrasts, respectively. Motor and locomotor activity were analyzed using ANOVA procedures followed by Dunnett's test. Continuous homogeneous neuropathology data were analyzed using ANOVA, followed by Dunnett's test while continuous non-homogeneous data were analyzed using the non-parametric Kruskall-Wallis test followed by a Mann-Whitney U Test for pair-wise comparisons. Micropathology frequency data were analyzed using a Chi-Square Test followed by a one-tailed Fisher's Exact Test.

C. Methods / Observations:

1. Mortality and Clinical Observations: Animals were observed at least once daily for mortality and morbidity. Detailed clinical observations were recorded daily.

2. Body weight: Animals were weighed weekly as a component of the FOB.

3. Food consumption: Food consumption was not recorded. The test substance was administered by gavage in a single dose.

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4. Cholinesterase Determination: N/A

5. Neurobehavioral Assessment:

5a. Functional Observational Battery (FOB): The same technicians were used throughout testing. Observations for all animals were performed by the same observer throughout the study with a second person performing measurements such as grip strength and foot splay. Technicians were blind with respect to group assignment of an individual animal. The test room was a standard animal room. The animals were allowed to acclimate in this room for at least 30 minutes. This room was maintained on the same light/dark cycle and settings for temperature and relative humidity as the animal room with testing conducted during the light phase. The FOB began approximately 4 hours following treatment with motor activity assessments concluding approximately 6 hours following treatment. Environmental conditions during FOB were not described. Scoring criteria were given for the measured parameters. For open field observations, each rat was placed in the center of a flat surface with a perimeter barrier for 2 minutes. No information on the equipment used in measuring grip strength was presented in the test report. A thermistor was used to measure colonic temperature.

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The checked (X) parameters were examined.

	HOME CAGE OBSERVATIONS		**HANDLING OBSERVATIONS**		**OPEN FIELD OBSERVATIONS**
X	Posture*	X	Reactivity*	X	Mobility
	Biting	X	Lacrimation* / chromodacryorrhea	X	Rearing+
X	Convulsions*	X	Salivation*	X	Arousal/gereral activity level*
X	Tremors*		Piloerection*	X	Convulsions*
X	Abnormal Movements*		Fur appearance	X	Tremors*
	Palpebral closure*	X	Palpebral closure*	X	Abnormal movements*
X	Piloerection		Respiratory rate+	X	Urination / defecation*
X	Gait Abnormalities	X	Red/crusty deposits*	X	Grooming
	SENSORY OBSERVATIONS		Mucous membranes /eye /skin colour	X	Gait abnormalities / posture*
X	Approach response+	X	Eye prominence*	X	Gait score*
X	Touch response+	X	Muscle tone*	X	Bizarre / stereotypic behaviour*
X	Startle response*			X	Backing
X	Pain response*				Time to first step
X	Pupil response*				
	Eyeblink response		**PHYSIOLOGICAL OBSERVATIONS**		**NEUROMUSCULAR OBSERVATIONS**
	Forelimb extension	X	Body weight*		Hindlimb extensor strength
	Hindlimb extension	X	Body temperature+	X	Forelimb grip strength*
X	Air righting reflex+			X	Hindlimb grip strength*
	Olfactory orientation			X	Landing foot splay*
X	Pupil size		**OTHER OBSERVATIONS**		Rotarod performance

*Required parameters; +Recommended parameters

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5b. Locomotor Activity: The FOB began approximately 4 hours following treatment with motor activity assessments beginning approximately 30 minutes after the cessation of the FOB and concluding approximately 6 hours following treatment. Testing was staggered over a 4-day interval with males and females tested on separate days. Motor and locomotor activity was evaluated by testing the animals individually in a figure-eight maze that had 8 infrared emitter/detector pairs. Evaluations were conducted for 10-minute intervals for 60 minutes. Motor activity was defined as the number of beam interruptions that occurred during the test session. Locomotor activity was defined as the number of beam interruptions minus consecutive counts for a given beam. Also, habituation, which was defined as a decrement in activity during the test session, was measured.

6. Sacrifice and Pathology: A complete gross necropsy was performed on all animals. The necropsy involved an examination of all organs, body cavities, cut surfaces, external orifices, and surfaces. On day 14, the first 6 males and 6 females at each dose level were selected for perfusion and collection of tissues. If perfusion was inadequate, one of the remaining animals was used. Animals selected for perfusion were deeply anesthetized using an intraperitoneal dose of 50 mg/kg of pentobarbital and then perfused via the left ventricle with a sodium nitrite (in phosphate buffer) flush followed by universal fixative (4% EM-grade formalin and 1% glutaraldehyde). The entire brain and spinal cord, both eyes with optic nerves, and selected (bilateral) peripheral nerves (sciatic, tibial, and sural), the gasserian ganglion, gastrocnemius muscle, and physical identifier were dissected from each animal and post-fixed in 10% buffered formalin. The brain was weighed upon removal from the skull, prior to placement into formalin, and the brain:body weight ratio was calculated. Surviving animals not perfused were sacrificed by CO_2 asphyxiation. Tissues from the control and high-dose group animals and any gross lesions collected at necropsy were processed for microscopic examination. 8 coronal sections of the brain and sections from 3 levels of the spinal cord (cervical, thoracic, and lumbar) were embedded in paraffin, sectioned at approximately 5 µm, and examined utilizing hematoxylin and eosin, luxol fast blue/cresyl violet and Sevier-Munger stains with sections collected from each level. Dorsal root ganglia (including dorsal and ventral root fibers) from the cervical and lumbar swellings, gasserian ganglion, eyes, optic nerves, and gastrocnemius muscle were embedded in glycol methacrylate. Peripheral nerve tissues (sciatic, tibial, and sural nerves) were embedded in glycol methacrylate and cut in longitudinal section. The sciatic nerve was also cut in cross-section. Glycol methacrylate-embedded tissues were sectioned at 2-3 µm and stained using hematoxylin and eosin. Microscopic evaluation included the following major brain regions: olfactory bulbs (Level 1), cerebral cortex (Levels 2 to 6), caudate-putamen/globus pallidus (Levels 2 to 5), hippocampus (Levels 5 to 6), thalamus (Levels 4 to 5), hypothalamus (Levels 4 to 5), midbrain including tectum, tegmentum, and cerebral peduncles (Level 6), cerebellum (Levels 7 to 8), and medulla oblongata (Levels 7 to 8).

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The checked (X) tissues were evaluated.

	CENTRAL NERVOUS SYSTEM	X	**PERIPHERAL NERVOUS SYSTEM**
	BRAIN		**SCIATIC NERVE**
X	Forebrain		Mid-thigh
X	Center of cerebrum		Sciatic Notch
X	Midbrain	X	Sciatic nerve (uncharacterized
X	Cerebellum		**OTHER**
	Pons	X	Sural Nerve
X	Medulla oblongata	X	Tibial Nerve
	SPINAL CORD		Peroneal Nerve
X	Cervical swelling	X	Lumbar dorsal root ganglion
X	Lumbar swelling	X	Lumbar dorsal root fibers
	Thoracic swelling	X	Lumbar ventral root fibers
	OTHER	X	Cervical dorsal root ganglion
X	Gasserian Ganglion	X	Cervical dorsal root fibers
	Trigeminal nerves	X	Cervical ventral root fibers
X	Optic nerve		
X	Eyes		
X	Gastrocnemius muscle		

7. Positive Controls: The positive control data cited were collected over seven years prior to the time interval of the study in review.

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II. Results

Findings:

General observation: There were no compound-related deaths occurred at any dose level. Compound-related clinical signs were evident in the **initial** study at all dose levels. Urine stain was observed at all treated dose levels and was considered to be related to treatment. Perianal stain was observed in males (one each) at the two highest dose levels but not at the lowest dose or in females at any dose level. These signs were evident on day 0 and generally resolved within 1 - 4 days after treatment. For the **follow-up** study, urine stain was evident in females treated with 500 mg/kg, but not at lower levels or in males at any dose level. This sign was first observed on day 0 and resolved within 1-4 days after treatment. Based on these results, the NOAEL for clinical signs is 100 mg/kg for males and females. Body weight was not affected by treatment in males or females at any dose.

Neurobehavioral tests (FOB, MA, LMA): For the functional observational battery (FOB) in the **initial** study, urine stain was observed in males (one each) at the low-and mid-dose levels. There were no findings related to treatment observed in high-dose males or females at any dose level. In the **follow-up** study, there were no treatment-related findings at any dose level in either sex. Based on these results, the NOAEL for functional observations is 100 mg/kg for males and 2,000 mg/kg for females.

Measures of motor and locomotor activity in the **initial** study were significantly reduced at 2,000 mg/kg in males (48 % and 64 %, respectively) and females (40 % and 65 %, respectively). At 500 mg/kg, motor activity was reduced (32 %) and locomotor activity was significantly reduced (44 %) in males only. Locomotor activity was slightly reduced (29 %) in males of the low dose group (200 mg/kg). In addition, interval motor and locomotor activity was reduced in males at several intervals at 500 and 2,000 mg/kg and for locomotor in males at 200 mg/kg. A similar trend was evident in females at 500 and 2,000 mg/kg (locomotor only at middose), but the difference from control was not statistically significant. In the **follow-up** study, motor and locomotor activity was slightly reduced (21 % and 30 %, respectively) in males dosed at 500 mg/kg, but not in lower levels (50 and 100 mg/kg) or in females at any dose. Interval motor and locomotor activity was reduced at several intervals in both sexes at 500 mg/kg. Based on these measures of activity, the NOAEL for motor/locomotor activity is 100 mg/kg for males and 200 mg/kg for females. There were no compound-related effects on days 7 or 14 after treatment and habituation was not affected by treatment on any day, at any dose.

Gross pathology, brain weight, histopathology: There were no compound-related gross lesions in males or females at any dose. The brain weight was not affected by treatment in both sexes at any dose. Compound-related microscopic lesions were not evident in high-dose males or females at 2,000 mg/kg bw.

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II. Conclusion: In the initial study evidence of acute oral toxicity was observed in both sexes at 200, 500 and 2,000 mg/kg bw. Evidence of toxicity was limited to clinical signs (urine stain in all doses, perianal stain in males at 500 and 2,000 mg/kg) and decreased activity in the figure-eight maze (in females at 500 and 2,000 mg/kg and in males at 200, 500 and 2,000 mg/kg) beginning on the day of treatment and with complete recovery by day 7. There was no evidence of neurotoxicity at any dose and there were no compound-related gross or microscopic lesions at a limit dose of 2,000 mg/kg bw. A follow-up study confirmed findings in the main study at 500 mg/kg bw, with no compound-related effects at 50 and 100 mg/kg.
Based on these results, the overall NOAEL for BYI 08330 by acute oral administration is 100 mg/kg for males and females.

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IIA 5.7.2 Delayed neurotoxicity following acute exposure

As BYI 08330 does not belong to the class of organophosphates, such testing was not necessary.

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IIA 5.7.3 28-day delayed neurotoxicity

See IIA 5.7.2

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IIA 5.7.4 Subchronic neurotoxicity - rat - 90-day

As BYI 08330 did not show any evidence of a neurotoxic potential in short- and long-term studies as well as in an acute neurotoxicity screening study, such testing was not necessary.

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IIA 5.7.5 Postnatal developmental neurotoxicity

Not required.

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IIA 5.8 Toxicity studies on metabolites

Acute (oral rat) and genotoxicity (bacterial reverse mutation) testing was conducted on the following four plant metabolites of BYI 08330 exceeding 0.01 mg/kg raw foodstuff for human consumption or exceeding 0.05 mg/kg raw animal fodder:

1. BYI 08330-cis-ketohydroxy
2. BYI 08330-desmethyl-ketohydroxy
3. BYI 08330-mono-hydroxy
4. BYI 08330-di-hydroxy

In the ADME study (Report No. MEF048/04), these plant metabolites were not identified in the excreta of the rat or were identified in the excreta to a minor degree only: However, in a later supplemental metabolism study in the rat (Report No. MEF 06/328), BYI 08330-cis-ketohydroxy was identified as the second prominent metabolite (beside the enol) in liver and kidneys after a single oral administration of 2 mg/kg bw, whereas BYI 08330-cis-ketohydroxy was only present at trace levels in plasma, and urine. In addition, BYI 08330-desmethyl-ketohydroxy was also detected in liver and kidneys in this study.
A supportive low dose ADME study (Report No. MEF-06/007 with radiolabelled BYI 08330-cis-ketohydroxy in male rats, revealed a complete metabolization mainly to BYI 08330-desmethyl-ketohydroxy followed by single and multiple oxidation steps resulting in different mono-, di- and tri-oxygenated metabolites which are probably represented by isomers of mono-hydroxy, di-hydroxy and tri-hydroxy metabolites. Thus, there is no toxicological concern for the BYI 08330-cis-ketohydroxy residues and BYI 08330-desmethyl-ketohydroxy residues found in plants because these plant metabolites were formed in relevant amounts in rats and have therefore been co-tested in the toxicity studies with BYI 08330.

With all four metabolites, the results of the acute oral study (LD50 > 2,000 mg/kg) and the bacterial reverse mutation test (negative = no evidence of mutagenicity) were equivalent to those observed with the parent compound and uncritical.

Furthermore, all of these plant metabolites were of structural similarity to the main rat metabolites (BYI 08330-enol and BYI 08330-desmethylenol) assuming no additional human health hazard.
No other toxicity studies were deemed necessary as potential consumer exposure of these plant residues was calculated to be clearly below 1 µg/kg bw/day.

Toxicity tests were not performed with the plant metabolite BYI 08330-enol-glucoside. An additional single low dose toxicokinetic study (Report No. MEF-06/006) implies that the metabolism of BYI 08330-enol-glucoside follows the same metabolic pathways as the one of the aglycone BYI 08330-enol and the parent compound (BYI 08330).

Therefore, all plant metabolites are considered to be toxicologically adaequately investigated and uncritical for human health.

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BYI 08330-CIS-ketohydroxy

Acute oral toxicity

Report:	**KIIA 5.8/01, [redacted]., 2005**
Title:	BYI 08330-CIS-Ketohydroxy – Acute toxicity in the rat after oral administration
Report No & Document No	AT02506 M-258306-01-2
Guidelines:	OECD 423, EEC Directive 67/548 B.1.tris, US EPA OPPTS 870.1100. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In an acute oral toxicity study, two groups of three fasted, young adult female Wistar [HsdCpb:Wu] rats were given a single oral dose (gavage) of 2,000 mg/kg of BYI 08330-CIS-ketohydroxy, batch no. NLL7549-7, purity 98.7 %, formulated in tap water with the aid of 2 % Cremophor EL.
The application volume was 10 mL/kg bw. The post observation period was 14 day. The animals were evaluated for the effect of the test compound on body weight, and clinical signs.
BYI 08330-CIS-ketohydroxy was found to have virtually no acute oral toxicity following exposure of rats. Clinical signs were not observed during the course of the study and there were no effects on body weight development. On the basis of this study, the result with BYI 08330-CIS-ketohydroxy was considered equivalent to that observed with the parent compound.

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I. Material and methods

1. Test Material:	BYI 08330-CIS-ketohydroxy
Description:	light yellow solid
Chemical name:	3-(2, 5-dimethy lphenyl)-3-hydroxy-8-methoxyl-azaspiro[4.5]decane-2,4-dione
Lot/Batch No.:	NLL 7549-7
Purity:	98.7%
2. Vehicle and/or positive control:	Tap water with the aid of 2% Cremophor EL
3. Test animals:	
Species:	Rat, females
Strain:	Wistar [HsdCpb:Wu]
Age/weight at study initiation:	10-12 weeks / 141 g – 165 g
Source:	[redacted] NL
Acclimatisation:	At least 5 days
Housing:	The animals were group caged conventionally in polycarbonate cages.
Diet:	[redacted] 3883.0.15
Water:	Tap water *ad libitum*
Environmental conditions:	**Temperature and** 22 ± 2°C
	Humidity: 55 ± 5%
	Lighting: 12 hours rhythm
	Air changes: approx. 10 changes per hour

B. Study design and methods:

1. In life dates: 2005-08-17 – 2005-09-14

2. Animal assignment and treatment: The animals were assigned to their groups by randomization. The test substance was administered in a single oral dose by gavage. The volume administered was 10 ml/kg bw. For administration, food was withheld from the animals for approximately 16 - 24 h before administration of the test compound, and they were fed again approximately 2 - 4 h after administration.

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II. Results and discussion:

Mortality: Mortalities were not observed.

Table 5.8-1: Doses, mortality / animals treated

Dose [mg/kg bw]	Toxicological results#			Duration of signs	Time of death	Mortality [%]
Females						
2,000 (1st)	0	0	3	--	--	0
2,000 (2nd)	0	0	3	--	--	0
LD_{50}: >2,000 mg/kg bw						

1st figure = number of dead animals; 2nd figure = number of animals with signs; 3rd figure = number of animals in the group

The acute oral LD_{50} of BYI 08330-CIS-ketohydroxy was > 2,000 mg/kg bw. According to OECD guideline 423, the LD_{50} can be classified as ≥5,000 mg/kg.

Body weight: No treatment-related findings.

Clinical signs: No treatment-related findings.

Necropsy: No treatment-related findings.

III. Conclusion: BYI 08330-CIS-ketohydroxy has no acute oral toxicity in rats.
Classification/labelling according to Commission Directive 67/548/EEC: none

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Genotoxicity

Report:	**KIIA 5.8/02, ; 2005**
Title:	BYI 08330-CIS-Ketohydroxy – Salmonella/microsome test plate incorporation and preincubation method
Report No & Document No	AT02735 M-262850-01-3
Guidelines:	OECD Guideline No. 471, EEC B.13/14, US EPA 712-C-98-247 OPPTS 870.5100. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a reverse gene mutation assay in bacteria, histidine-auxotrophic Salmonella typhimurium LT2 mutant strains TA 100, TA 1535, TA 98, TA 1537 and TA 102 were exposed to BYI 08330-CIS-ketohydroxy, batch no. NLL7549-7, purity 98.7 %, using dimethylsulfoxide (DMSO) solvent. The Salmonella/microsome plate incorporation test was initially investigated for point mutagenic effects at doses of 0, 16, 50, 158, 500, 1,581 and 5,000 µg/plate on the five Salmonella strains with and without S9 mix. The independent repeat was performed as preincubation for 20 minutes at 37°C.

Doses up to and including 1,581 µg/plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At 5,000 µg/plate, the substance had only in the preincubation trial a strong, strain-specific bacteriotoxic effect, so that this dose could not be used for assessment purposes. In the plate incorporation trial 5,000 µg/plate could still be used for assessment. No substance precipitation occurred. Evidence of mutagenic activity of BYI 08330 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls had a marked mutagenic effect.

Therefore, BYI 08330-CIS-ketohydroxy was considered to be non-mutagenic without and with S9 mix in the plate incorporation as well as in the preincubation modification of the Salmonella/ microsome test.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330-cis-ketohydroxy (metabolite of BYI 08330)

Description:	white powder, room temp storage
Lot/Batch No.:	NLL7549-7
Purity (ai%):	98.7% (27-Jun-05)
CAS No. of TGAI:	unavailable
Name / Structure:	3-(2,5-dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione
Solvent Used:	DMSO

2. Control Materials:

Negative:	N/A	
Solvent (final conc'n):	DMSO, (0.1 ml/plate)	
Positive:	**Nonactivation**:	
	Sodium azide	10 µg/plate TA1535
	Nitrofurantoin	0.2 µg/plate TA 100
	4-Nitro-1, 2- phenylene diamine	0.5 µg/plate TA 98
	4-Nitro-1, 2- phenylene diamine	10 µg/plate TA1537
	Mitomycin C	0.2 µg/plate TA 102
	Cumene hydroperoxide	50 µg/plate TA 102
	Activation: 2-Aminoanthracene	3 µg/plate, all strains

3. Activation: S9 derived from

x induced	x Aroclor 1254	x Rat	x Liver
non-induced	Phenobarbitol	Mouse	Lung
	None	Hamster	Other
	Other	Other	

Describe S9 mix composition:

Cofactor solution: $MgCl_2$ x 6 H_2O	162.6 mg 70 mls, S9 fraction, 10 ml, KCl 0.15 M, 20 ml
KCl	246.0 mg
Glucose-6-phospate, disodium salt	179.1 mg
NADP, disodium salt	315.0 mg
Phosphate buffer	100.0 mM

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4. Test organisms: ***S. typhimurium*** **strains**

TA97	x TA98	x TA100	x TA102	TA104
x TA1535	x TA1537	TA1538	list any others	
Properly maintained?			x Yes	No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?			x Yes	No

5. Test compound concentrations used:

Nonactivated conditions: 0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment
Activated conditions: 0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment

B. Test performance

1. Type of *Salmonella* assay:
x standard plate test
x pre-incubation (20 minutes)
__ "Prival" modification *(i.e. azo-reduction method)*
__ spot test
__ other

2. Protocol
In the first trial, a plate incorporation method was employed (Ames *et al.* (1973) *Proc. Nat. Acad. Sci.* 70: 2281-2285, Ames *et al.* (1975) *Mutation Res.* 31: 347-364). For the mutant count, three plates were used, both with and without S9 mix, for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control. Each positive control also contained three plates per strain. The amount of solvent for the test substance and for the controls was 0.1 ml/plate. The doses for the first trial were routinely determined on the basis of a standard protocol: if not limited by solubility 5,000 µg or 5 µl per plate were used as the highest dose. At least five additional doses were routinely used. If less than three doses were used for assessment, at least used for assessment, at least two repeats were performed. The results of the first experiment were then considered as a pre-test for toxicity. However, in case of a positive response or if at least three doses could be used for assessment, the first trial was included in the assessment. If the second test confirmed the results of the first, no additional repeat was performed. Doses of repeats were chosen on the basis of the results obtained in the first experiment. The independent repeat was performed as preincubation 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. For the mutant count, three plates were used for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control.

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Each positive control also contained three plates per strain. The doses of this trial were determined on the basis of the results of the plate incorporation assay. Doses are given as [µg/tube for better separation of plate incorporation and preincubation trials, despite the fact that µg/plate and µg/tube could be used synonymously. The toxicity of the substance was assessed in three ways. The first method was a gross appraisal of background growth on the plates for mutant determination. If a reduction in background growth was observed, it was indicated in the tables by the letter "b" after the mutant count. Where only a single "b", without any other values, is noted for a concentration, this "b" represents three plates with reduced background growth. (The same applies to the signs "c", "v", "p", "n" or"%", which may also be used in the tables). Secondly, a toxic effect of the substance was assumed when there was a marked and dose-dependent reduction in the mutant count per plate, compared to the negative controls. Thirdly, the titer was determined. Total bacterial counts were taken on two plates for each concentration studied with S9 mix. However, if an evaluation was performed only without S9 mix, the bacterial count was taken without S9 mix. The bacterial suspensions were obtained from 17-hour cultures in nutrient broth, which had been incubated at 37°C and 90 rpm. These suspensions were used for the determination of mutant counts. No standardized procedure was employed to set the bacterial suspensions at a defined density of viable cells per milliliter, since the chosen method of incubation normally produces the desired density. However, the numbers of viable cells were established in a parallel procedure by determining the titers. The dilution of bacterial suspensions used for the determination of titers was 1:1,000,000. Titers were determined under the same conditions as were the mutations, except that the histidine concentration in the soft agar was increased five-fold to permit the complete growth of bacteria. The tests were performed both with and without S9 mix. The count was made after the plates had been incubated for 48 hours at 37°C. If no immediate count was possible, plates were temporarily stored in a refrigerator.

3. Statistical Analysis: No statistical analysis was performed on the study data.

4. Evaluation Criteria:

The following criteria determined the acceptance of an assay:

a) The negative controls had to be within the expected range, as defined by published data and/ or the laboratories' own historical data; b) the positive controls had to show sufficient effects, as defined by the laboratories' experience; and c) titer determinations had to demonstrate sufficient bacterial density in the suspension. Only trials which complied with all three of the above criteria were accepted for assessment. Even if the criteria for points (b) and (c) were not met, a trial was accepted if it showed mutagenic activity of the test compound. Furthermore, an unacceptable trial would have been repeated.

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II. Results and Discussions:

Findings:
Doses up to and including 1,581 µg/plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At 5,000 µg/plate, the substance had only in the preincubation trial a strong, strain-specific bacteriotoxic effect, so that this dose could not be used for assessment purposes. In the plate incorporation trial 5,000 µg/plate could still be used for assessment. No substance precipitation occurred. Evidence of mutagenic activity of BYI 08330 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls had a marked mutagenic effect.

III. Conclusions: BYI 08330-CIS-ketohydroxy was considered to be non-mutagenic without and with metabolic activation under the conditions of the Salmonella/microsome test.

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BYI 08330-desmethyl-ketohydroxy

Acute oral toxicity

Report:	**KIIA 5.8/03, , 2006**
Title:	BYI 08330-desmethyl-ketohydroxy – Acute toxicity in the rat after oral administration
Report No & Document No	AT02927 M-269279-01-2
Guidelines:	OECD 423, EEC Directive 67/548 B.1.tris, US EPA OPPTS 870.110. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In an acute oral toxicity study, two groups of three fasted, young adult female Wistar [HsdCpb:Wu] rats were given a single oral dose (gavage) of 2,000 mg/kg of BYI 08330-desmethyl-ketohydroxy, batch no. KATH4726-5-1, purity 94.6 %, formulated in tap water with the aid of 2 % Cremophor EL. The application volume was 10 mL/kg bw. The post observation period was 14 day. The animals were evaluated for the effect of the test compound on body weight, and clinical signs.
BYI 08330-desmethyl-ketohydroxy was found to have virtually no acute oral toxicity following exposure of rats. Clinical signs were not observed during the course of the study and there were no effects on body weight development. On the basis of this study, the result with BYI 08330-desmethyl-ketohydroxy was considered equivalent to that observed with the parent compound.

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I. Material and methods

1. Test Material: BYI 08330-desmethyl-ketohydroxy

- **Description:** light beige powder
- **Chemical name:** (5s,8s)-3-(2,5-dimethylphenyl)-3,8-dihydroxy-1-azaspiro[4.5]decan-2-one
- **Lot/Batch No.:** KATH 4726-5-1
- **Purity:** 94.6%

2. Vehicle and/or positive control: Tap water with the aid of 2% Cremophor EL

3. Test animals:

- **Species:** Rat, females
- **Strain:** Wistar [HsdCpb:Wu]
- **Age/weight at study initiation:** 10-12 weeks / 180 g – 194 g
- **Source:** Germany
- **Acclimatisation:** At least 5 days
- **Housing:** The animals were group caged conventionally in polycarbonate cages.
- **Diet:** 3883.0.15
- **Water:** Tap water *ad libitum*
- **Environmental conditions:**
  - **Temperature** 22 ± 2°C
  - **and Humidity:** 55 ± 5%
  - **Lighting:** 12 hours rhythm
  - **Air changes:** approx. 10 changes per hour

B. Study design and methods:

1. In life dates: 2006-03-02 – 2006-03-22

2. Animal assignment and treatment: The animals were assigned to their groups by randomization. The test substance was administered in a single oral dose by gavage. The volume administered was 10 ml/kg bw. For administration, food was withheld from the animals for approximately 16 - 24 h before administration of the test compound, and they were fed again approximately 2 - 4 h after administration.

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II. Results and discussion:

Mortality: Mortalities were not observed.

Table 5.8-2: Doses, mortality / animals treated

Dose [mg/kg bw]	Toxicological results#			Duration of signs	Time of death	Mortality [%]
Females						
2,000 (1st)	0	0	3	--	--	0
2,000 (2nd)	0	0	3	--	--	0
LD_{50}: >2,000 mg/kg bw						

1st figure = number of dead animals; 2nd figure = number of animals with signs; 3rd figure = number of animals in the group

The acute oral LD_{50} of BYI 08330-desmethyl-ketohydroxy was > 2,000 mg/kg bw. According to OECD guideline 423, the LD_{50} can be classified as ≥5,000 mg/kg.

Body weight: No treatment-related findings.

Clinical signs: No treatment-related findings.

Necropsy: No treatment-related findings.

III. Conclusion: BYI 08330-desmethyl-ketohydroxy has no acute oral toxicity in rats.
Classification/labelling according to Commission Directive 67/548/EEC: none

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<u>Genotoxicity</u>

Report:	**KIIA 5.8/04, [redacted].; 2006**
Title:	BYI 08330-desmethyl-ketohydroxy– Salmonella/microsome test plate incorporation and preincubation method
Report No & Document No	AT03027 M-271090-01-2
Guidelines:	OECD Guideline No. 471, EEC B.13/14, US EPA 712-C-98-247 OPPTS 870.5100. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a reverse gene mutation assay in bacteria, histidine-auxotrophic Salmonella typhimurium LT2 mutant strains TA 100, TA 1535, TA 98, TA 1537 and TA 102 were exposed to BYI 08330-desmethyl-ketohydroxy, batch no. KATH4726-5-1, purity 94.6 %, using dimethylsulfoxide (DMSO) solvent. The Salmonella/microsome plate incorporation test was initially investigated for point mutagenic effects at doses of 0, 16, 50, 158, 500, 1,581 and 5,000 µg/plate on the five Salmonella strains with and without S9 mix. The independent repeat was performed as preincubation for 20 minutes at 37°C.
Total bacteria counts remained unchanged and no inhibition of growth was observed. At higher doses, the substance had only a weak, strain-specific bacteriotoxic effect. Due to the weakness of this effect this range could nevertheless be used up to 5,000 µg/plate for assessment purposes. Evidence of mutagenic activity of BYI 08330-desmethyl-ketohydroxy was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls had a marked mutagenic effect.

Therefore, BYI 08330-desmethyl-ketohydroxy was considered to be non-mutagenic without and with metabolic activation under the conditions of the Salmonella/microsome test.

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I. Materials and methods

A. Materials:

1. Test Material: BYI 08330-desmethyl-ketohydroxy (metabolite of BYI 08330)

Description:	white powder, room temp storage
Lot/Batch No.:	KATH 4726-5-1
Purity (ai%):	94.6% (05-Jan-06)
CAS No. of TGAI:	203313-25-1
Name / Structure:	(5s,8s)-3-(2,5-dimethylphenyl)-3,8-dihydroxy-1-azaspiro[4.5]decan-2-one
Solvent Used:	DMSO

2. Control Materials:

Negative:	N/A	
Solvent (final conc'n):	DMSO (0.1 ml/plate)	
Positive:	**Nonactivation:**	
	Sodium azide	10 µg/plate TA1535
	Nitrofurantoin	0.2 µg/plate TA 100
	4-Nitro-1, 2- phenylene diamine	0.5 µg/plate TA 98
	4-Nitro-1, 2- phenylene diamine	10 µg/plate TA1537
	Mitomycin C	0.2 µg/plate TA 102
	Cumene hydroperoxide	50 µg/plate TA 102
	Activation: 2-Aminoanthracene	3 µg/plate, all strains

3. Activation: S9 derived from

x induced	x Aroclor 1254	x Rat	x Liver
non-induced	Phenobarbitol	Mouse	Lung
	None	Hamster	Other
	Other	Other	

Describe S9 mix composition:

Cofactor solution: $MgCl_2$ x 6 H_2O	162.6 mg 70 mls, S9 fraction, 10 ml, KCl 0.15 M, 20 ml
KCl	246.0 mg
Glucose-6-phospate, disodium salt	179.1 mg
NADP, disodium salt	315.0 mg
Phosphate buffer	100.0 mM

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4. Test organisms: ***S. typhimurium*** **strains**

	TA97	x TA98	x TA100	x TA102	TA104
	x TA1535	x TA1537	TA1538	list any others	
Properly maintained?				x Yes	No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?				x Yes	No

5. Test compound concentrations used:

Nonactivated conditions: 0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment
Activated conditions: 0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment

B. Test performance

1. Type of *Salmonella* assay:
x_ standard plate test
x_ pre-incubation (20_ minutes)
__ "Prival" modification *(i.e. azo-reduction method)*
__ spot test
__ other

2. Protocol
In the first trial, a plate incorporation method was employed (Ames *et al.* (1973) *Proc. Nat. Acad. Sci.* 70: 2281-2285, Ames *et al.* (1975) *Mutation Res.* 31: 347-364). For the mutant count, three plates were used, both with and without S9 mix, for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control. Each positive control also contained three plates per strain. The amount of solvent for the test substance and for the controls was 0.1 ml/plate. The doses for the first trial were routinely determined on the basis of a standard protocol: if not limited by solubility 5,000 µg or 5 µl per plate were used as the highest dose. At least five additional doses were routinely used. If less than three doses were used for assessment, at least used for assessment, at least two repeats were performed. The results of the first experiment were then considered as a pre-test for toxicity. However, in case of a positive response or if at least three doses could be used for assessment, the first trial was included in the assessment. If the second test confirmed the results of the first, no additional repeat was performed. Doses of repeats were chosen on the basis of the results obtained in the first experiment. The independent repeat was performed as preincubation 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. For the mutant count, three plates were used for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control.

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Each positive control also contained three plates per strain. The doses of this trial were determined on the basis of the results of the plate incorporation assay. Doses are given as µg/tube for better separation of plate incorporation and preincubation trials, despite the fact that µg/plate and µg/tube could be used synonymously. The toxicity of the substance was assessed in three ways. The first method was a gross appraisal of background growth on the plates for mutant determination. If a reduction in background growth was observed, it was indicated in the tables by the letter "b" after the mutant count. Where only a single "b", without any other values, is noted for a concentration, this "b" represents three plates with reduced background growth. (The same applies to the signs "c", "v", "p", "n" or"%", which may also be used in the tables). Secondly, a toxic effect of the substance was assumed when there was a marked and dose-dependent reduction in the mutant count per plate, compared to the negative controls. Thirdly, the titer was determined. Total bacterial counts were taken on two plates for each concentration studied with S9 mix. However, if an evaluation was performed only without S9 mix, the bacterial count was taken without S9 mix. The bacterial suspensions were obtained from 17-hour cultures in nutrient broth, which had been incubated at 37°C and 90 rpm. These suspensions were used for the determination of mutant counts. No standardized procedure was employed to set the bacterial suspensions at a defined density of viable cells per milliliter, since the chosen method of incubation normally produces the desired density. However, the numbers of viable cells were established in a parallel procedure by determining the titers. The dilution of bacterial suspensions used for the determination of titers was 1:1,000,000. Titers were determined under the same conditions as were the mutations, except that the histidine concentration in the soft agar was increased five-fold to permit the complete growth of bacteria. The tests were performed both with and without S9 mix. The count was made after the plates had been incubated for 48 hours at 37°C. If no immediate count was possible, plates were temporarily stored in a refrigerator.

3. Statistical Analysis: No statistical analysis was performed on the study data.

4. Evaluation Criteria:

The following criteria determined the acceptance of an assay:

a) The negative controls had to be within the expected range, as defined by published data and/ or the laboratories' own historical data; b) the positive controls had to show sufficient effects, as defined by the laboratories' experience; and c) titer determinations had to demonstrate sufficient bacterial density in the suspension. Only trials which complied with all three of the above criteria were accepted for assessment. Even if the criteria for points (b) and (c) were not met, a trial was accepted if it showed mutagenic activity of the test compound. Furthermore, an unacceptable trial would have been repeated.

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II. Results and Discussions:

Findings:

Total bacteria counts remained unchanged and no inhibition of growth was observed. At higher doses, the substance had only a weak, strain-specific bacteriotoxic effect. Due to the weakness of this effect this range could nevertheless be used up to 5,000 µg/plate for assessment purposes. Evidence of mutagenic activity of BYI 08330-desmethyl-ketohydroxy was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant colonies compared to the corresponding negative controls.

III. Conclusions: BYI 08330-desmethyl-ketohydroxy was considered to be non-mutagenic without and with metabolic activation under the conditions of the Salmonella/microsome test.

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BYI 08330-mono-hydroxy

Acute oral toxicity

Report:	**KIIA 5.8/05, 2005**
Title:	BYI 08330-mono-hydroxy – Acute toxicity in the rat after oral administration
Report No & Document No	AT02687 M-262070-01-2
Guidelines:	OECD 423, EEC Directive 67/548 B.1.tris, US EPA OPPTS 870.110. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In an acute oral toxicity study, two groups of three fasted, young adult female Wistar [HsdCpb:Wu] rats were given a single oral dose (gavage) of 2,000 mg/kg of BYI 08330-mono-hydroxy, batch no. NLL7635-2D, purity 98.41 %, formulated in tap water with the aid of 2 % Cremophor EL. The application volume was 10 mL/kg bw. The post observation period was 14 day. The animals were evaluated for the effect of the test compound on body weight, and clinical signs.
BYI 08330-mono-hydroxy was found to have virtually no acute oral toxicity following exposure of rats. Clinical signs were not observed during the course of the study and there were no effects on body weight development. On the basis of this study, the result with BYI 08330-mono-hydroxy was considered equivalent to that observed with the parent compound.

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I. Material and methods

1. Test Material:	BYI 08330-mono-hydroxy
Description:	white powder
Chemical name:	3-(2,5-dimethylphenyl)-4-hydroxy-8-methoxy-1 -azaspiro [4.5] decan-2-one
Lot/Batch No.:	NLL 7635-2D
Purity:	98.41%
2. Vehicle and/or positive control:	Tap water with the aid of 2% Cremophor EL
3. Test animals:	
Species:	Rat, females
Strain:	Wistar [HsdCpb:Wu]
Age/weight at study initiation:	10-12 weeks / 171 g – 182 g
Source:	[redacted] Netherlands
Acclimatisation:	At least 5 days
Housing:	The animals were group caged conventionally in polycarbonate cages.
Diet:	[redacted] 3883.0.15
Water:	Tap water *ad libitum*
Environmental conditions:	**Temperature** 22 ± 2°C
	and Humidity: 55 ± 5%
	Lighting: 12 hours rhythm
	Air changes: approx. 10 changes per hour

B. Study design and methods:

1. In life dates: 2005-10-05 – 2005-10-26

2. Animal assignment and treatment: The animals were assigned to their groups by randomization. The test substance was administered in a single oral dose by gavage. The volume administered was 10 ml/kg bw. For administration, food was withheld from the animals for approximately 16 - 24 h before administration of the test compound, and they were fed again approximately 2 - 4 h after administration.

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II. Results and discussion:

Mortality: Mortalities were not observed.

Table 5.8-3: Doses, mortality / animals treated

Dose [mg/kg bw]	Toxicological results#			Duration of signs	Time of death	Mortality [%]
Females						
2,000 (1st)	0	0	3	--	--	0
2,000 (2nd)	0	0	3	--	--	0
LD_{50} : >2,000 mg/kg bw						

1st figure = number of dead animals; 2nd figure = number of animals with signs; 3rd figure = number of animals in the group

The acute oral LD_{50} of BYI 08330-mono-hydroxy was > 2,000 mg/kg bw. According to OECD guideline 423, the LD_{50} can be classified as ≥5,000 mg/kg.

Body weight: No treatment-related findings.

Clinical signs: No treatment-related findings.

Necropsy: No treatment-related findings.

III. Conclusion: BYI 08330-mono-hydroxy has no acute oral toxicity in rats.
Classification/labelling according to Commission Directive 67/548/EEC: none

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Genotoxicity

Report:	**KIIA 5.8/06, ; 2005**
Title:	BYI 08330-mono-hydroxy– Salmonella/microsome test plate incorporation and preincubation method
Report No & Document No	AT02716 M-262976-01-2
Guidelines:	OECD Guideline No. 471, EEC B.13/14, US EPA 712-C-98-247 OPPTS 870.5100. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a reverse gene mutation assay in bacteria, histidine-auxotrophic Salmonella typhimurium LT2 mutant strains TA 100, TA 1535, TA 98, TA 1537 and TA 102 were exposed to BYI 08330-mono-hydroxy, batch no. NLL7635-2D, purity 98.41 %, using dimethylsulfoxide (DMSO) solvent. The Salmonella/microsome plate incorporation test was initially investigated for point mutagenic effects at doses of 0, 16, 50, 158, 500, 1,581 and 5,000 µg/plate on the five Salmonella strains with and without S9 mix. The independent repeat was performed as preincubation for 20 minutes at 37°C.
Doses up to and including 5,000 µg/plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. Substance precipitation did not occur. Evidence of mutagenic activity of BYI 08330-mono-hydroxy was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls had a marked mutagenic effect.

Therefore, BYI 08330-mono-hydroxy was considered to be non-mutagenic without and with metabolic activation under the conditions of the Salmonella/microsome test.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330-mono-hydroxy

Description: white powder, room temp storage

Lot/Batch No.: NLL 7635-2D

Purity (ai%): 98.4% (26-Aug-05)

CAS No. of TGAI: unavailable

Name / Structure: 3-(2,5-dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]decan-2-one

Solvent Used: DMSO

2. Control Materials:

Negative: N/A

Solvent (final conc'n): DMSO (0.1 ml/plate)

Positive:

Nonactivation:		
Sodium azide		10 µg/plate TA1535
Nitrofurantoin		0.2 µg/plate TA 100
4-Nitro-1, 2- phenylene diamine		0.5 µg/plate TA 98
4-Nitro-1, 2- phenylene diamine		10 µg/plate TA1537
Mitomycin C		0.2 µg/plate TA 102
Cumene hydroperoxide		50 µg/plate TA 102
Activation:	2-Aminoanthracene	3 µg/plate, all strains

3. Activation: S9 derived from

x induced	x Aroclor 1254	x Rat	x Liver
non-induced	Phenobarbitol	Mouse	Lung
	None	Hamster	Other
	Other	Other	

Describe S9 mix composition:

Cofactor solution: $MgCl_2$ x 6 H_2O	162.6 mg	70 mls, S9 fraction, 10 ml, KCl 0.15 M, 20 ml
KCl	246.0 mg	
Glucose-6-phospate, disodium salt	179.1 mg	
NADP, disodium salt	315.0 mg	
Phosphate buffer	100.0 mM	

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4. Test organisms: *S. typhimurium* strains

	TA97	x TA98	x TA100	x TA102	TA104
	x TA1535	x TA1537	TA1538	list any others	

Properly maintained?	x Yes	No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	x Yes	No

5. Test compound concentrations used:

Nonactivated conditions: 0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment
Activated conditions: 0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment

B. Test performance

1. Type of *Salmonella* assay:
x_ standard plate test
x_ pre-incubation (20_ minutes)
__ "Prival" modification *(i.e. azo-reduction method)*
__ spot test
__ other

2. Protocol
In the first trial, a plate incorporation method was employed (Ames *et al.* (1973) *Proc. Nat. Acad. Sci.* 70: 2281-2285, Ames *et al.* (1975) *Mutation Res.* 31: 347-364). For the mutant count, three plates were used, both with and without S9 mix, for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control. Each positive control also contained three plates per strain. The amount of solvent for the test substance and for the controls was 0.1 ml/plate. The doses for the first trial were routinely determined on the basis of a standard protocol: if not limited by solubility 5,000 µg or 5 µl per plate were used as the highest dose. At least five additional doses were routinely used. If less than three doses were used for assessment, at least used for assessment, at least two repeats were performed. The results of the first experiment were then considered as a pre-test for toxicity. However, in case of a positive response or if at least three doses could be used for assessment, the first trial was included in the assessment. If the second test confirmed the results of the first, no additional repeat was performed. Doses of repeats were chosen on the basis of the results obtained in the first experiment. The independent repeat was performed as preincubation 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. For the mutant count, three plates were used for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control. Each positive control also contained three plates per strain. The doses of this trial were determined on the basis of the results of the plate incorporation assay. Doses are given as [µg/tube for better separation of plate incorporation and preincubation trials, despite the fact that µg/plate and µg/tube could be used synonymously.

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The toxicity of the substance was assessed in three ways. The first method was a gross appraisal of background growth on the plates for mutant determination. If a reduction in background growth was observed, it was indicated in the tables by the letter "b" after the mutant count. Where only a single "b", without any other values, is noted for a concentration, this "b" represents three plates with reduced background growth. (The same applies to the signs "c", "v", "p", "n" or"%", which may also be used in the tables).

Secondly, a toxic effect of the substance was assumed when there was a marked and dose-dependent reduction in the mutant count per plate, compared to the negative controls. Thirdly, the titer was determined. Total bacterial counts were taken on two plates for each concentration studied with S9 mix. However, if an evaluation was performed only without S9 mix, the bacterial count was taken without S9 mix. The bacterial suspensions were obtained from 17-hour cultures in nutrient broth, which had been incubated at 37°C and 90 rpm. These suspensions were used for the determination of mutant counts. No standardized procedure was employed to set the bacterial suspensions at a defined density of viable cells per milliliter, since the chosen method of incubation normally produces the desired density. However, the numbers of viable cells were established in a parallel procedure by determining the titers. The dilution of bacterial suspensions used for the determination of titers was 1:1,000,000. Titers were determined under the same conditions as were the mutations, except that the histidine concentration in the soft agar was increased five-fold to permit the complete growth of bacteria. The tests were performed both with and without S9 mix. The count was made after the plates had been incubated for 48 hours at 37°C. If no immediate count was possible, plates were temporarily stored in a refrigerator.

3. Statistical Analysis: No statistical analysis was performed on the study data.

4. Evaluation Criteria:

The following criteria determined the acceptance of an assay:

a) The negative controls had to be within the expected range, as defined by published data and/ or the laboratories' own historical data; b) the positive controls had to show sufficient effects, as defined by the laboratories' experience; and c) titer determinations had to demonstrate sufficient bacterial density in the suspension. Only trials which complied with all three of the above criteria were accepted for assessment. Even if the criteria for points (b) and (c) were not met, a trial was accepted if it showed mutagenic activity of the test compound. Furthermore, an unacceptable trial would have been repeated.

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II. Results and Discussions:

Findings:

Doses up to and including 5,000 µg/plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. Substance precipitation did not occur. Evidence of mutagenic activity of BYI 08330-mono-hydroxy was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls had a marked mutagenic effect.

III. Conclusions: BYI 08330-mono-hydroxy was considered to be non-mutagenic without and with metabolic activation under the conditions of the Salmonella/microsome test.

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BYI 08330-di-hydroxy

Acute oral toxicity

Report:	**KIIA 5.8/07, [redacted], 2006**
Title:	BYI 08330-di-hydroxy – Acute toxicity in the rat after oral administration
Report No & Document No	AT02995 M-270700-01-2
Guidelines:	OECD 423, EEC Directive 67/548 B.1.tris, US EPA OPPTS 870.110. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In an acute oral toxicity study, two groups of three fasted, young adult female Wistar [HsdCpb:Wu] rats were given a single oral dose (gavage) of 2,000 mg/kg of BYI 08330-di-hydroxy, batch no. KATH4727-3-6, purity 94.5 %, formulated in 0.5 % aqueous tylose. The application volume was 10 mL/kg bw. The post observation period was 14 day. The animals were evaluated for the effect of the test compound on body weight, and clinical signs.
BYI 08330-di-hydroxy was found to have virtually no acute oral toxicity following exposure of rats. Clinical signs were not observed during the course of the study and there were no effects on body weight development. On the basis of this study, the result with BYI 08330-di-hydroxy was considered equivalent to that observed with the parent compound.

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I. Material and methods

1. Test Material: BYI 08330-di-hydroxy

Description: light beige powder

Chemical name: (5s,8s)-3-(2,5-dimethylphenyl)-3,4-dihydroxy-8-methoxy-1-azaspiro[4.5]decan-2-one

Lot/Batch No.: KATH 4727-3-6

Purity: 94.5%

2. Vehicle and/or positive control: 0.5% aqueous tylose

3. Test animals:

Species: Rat, females

Strain: Wistar [HsdCpb:Wu]

Age/weight at study initiation: 10-12 weeks / 159 g – 191 g

Source: [redacted] Germany

Acclimatisation: At least 5 days

Housing: The animals were group caged conventionally in polycarbonate cages.

Diet: [redacted] 3883.0.15

Water: Tap water *ad libitum*

Environmental conditions:

Temperature	22 ± 2°C
and Humidity:	55 ± 5%
Lighting:	12 hours rhythm
Air changes:	approx. 10 changes per hour

B. Study design and methods:

1. In life dates: 2006-04-05 – 2006-04-26

2. Animal assignment and treatment: The animals were assigned to their groups by randomization. The test substance was administered in a single oral dose by gavage. The volume administered was 10 ml/kg bw. For administration, food was withheld from the animals for approximately 16 - 24 h before administration of the test compound, and they were fed again approximately 2 - 4 h after administration.

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II. Results and discussion:

Mortality: Mortalities were not observed.

Table 5.8-3: Doses, mortality / animals treated

Dose [mg/kg bw]	Toxicological results#			Duration of signs	Time of death	Mortality [%]
Females						
2,000 (1st)	0	0	3	--	--	0
2,000 (2nd)	0	0	3	--	--	0
LD_{50} : >2,000 mg/kg bw						

1st figure = number of dead animals; 2nd figure = number of animals with signs; 3rd figure = number of animals in the group

The acute oral LD_{50} of BYI 08330-di-hydroxy was > 2,000 mg/kg bw. According to OECD guideline 423, the LD_{50} can be classified as ≥5,000 mg/kg.

Body weight: No treatment-related findings.

Clinical signs: No treatment-related findings.

Necropsy: No treatment-related findings.

III. Conclusion: BYI 08330-di-hydroxy has no acute oral toxicity in rats.
Classification/labelling according to Commission Directive 67/548/EEC: none

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Genotoxicity

Report:	**KIIA 5.8/08, ; 2005**
Title:	BYI 08330-di-hydroxy– Salmonella/microsome test plate incorporation and preincubation method
Report No & Document No	AT03069 M-27980-01-2
Guidelines:	OECD Guideline No. 471, EEC B.13/14, US EPA 712-C-98-247 OPPTS 870.5100. Deviations: none
OECD/FIFRA GLPS	Yes (certified laboratory); Deviations: none

Executive Summary

In a reverse gene mutation assay in bacteria, histidine-auxotrophic Salmonella typhimurium LT2 mutant strains TA 100, TA 1535, TA 98, TA 1537 and TA 102 were exposed to BYI 08330-di-hydroxy, batch no. KATH 4727-3-6, purity 94.5 %, using dimethylsulfoxide (DMSO) solvent. The Salmonella/microsome plate incorporation test was initially investigated for point mutagenic effects at doses of 0, 16, 50, 158, 500, 1,581 and 5,000 µg/plate on the five Salmonella strains with and without S9 mix. The independent repeat was performed as preincubation for 20 minutes at 37°C.
Doses up to and including 5,000 µg/plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. Evidence of mutagenic activity of BYI 08330-di-hydroxy was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls had a marked mutagenic effect.

Therefore, BYI 08330-di-hydroxy was considered to be non-mutagenic without and with metabolic activation under the conditions of the Salmonella/microsome test.

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I. Material and methods

A. Materials:

1. Test Material: BYI 08330-di-hydroxy

Description: white powder, room temp storage

Lot/Batch No.: KATH 4727-3-6

Purity (ai%): 94.5% (07-Mar-06)

CAS No. of TGAI: unavailable

Name / Structure: (5s,8s)-3-(2,5-dimethylphenyl)-3,4-dihydroxy-8-methoxy-1-azaspiro[4.5]decan-2-one

Solvent Used: DMSO

2. Control Materials:

Negative: N/A

Solvent (final conc'n): DMSO, (0.1 ml/plate)

Positive: **Nonactivation**:

Sodium azide	10 µg/plate TA1535
Nitrofurantoin	0.2 µg/plate TA 100
4-Nitro-1, 2- phenylene diamine	0.5 µg/plate TA 98
4-Nitro-1, 2- phenylene diamine	10 µg/plate TA1537
Mitomycin C	0.2 µg/plate TA 102
Cumene hydroperoxide	50 µg/plate TA 102
Activation: 2-Aminoanthracene	3 µg/plate, all strains

3. Activation: S9 derived from

x induced	x Aroclor 1254	x Rat	x Liver
non-induced	Phenobarbitol	Mouse	Lung
	None	Hamster	Other
	Other	Other	

Describe S9 mix composition:

Cofactor solution: $MgCl_2$ x 6 H_2O	162.6 mg 70 mls, S9 fraction, 10 ml, KCl 0.15 M, 20 ml
KCl	246.0 mg
Glucose-6-phospate, disodium salt	179.1 mg
NADP, disodium salt	315.0 mg
Phosphate buffer	100.0 mM

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4. Test organisms: *S. typhimurium* strains

TA97	x TA98	x TA100	x TA102	TA104
x TA1535	x TA1537	TA1538	list any others	

Properly maintained?	x Yes	No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	x Yes	No

5. Test compound concentrations used:

Nonactivated conditions: 0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment
Activated conditions: 0, 16, 50, 158, 500, 1,581, 5,000 µg/plate, all strains, 3 replicates/treatment

B. Test performance

1. Type of *Salmonella* assay:
x standard plate test
x pre-incubation (20 minutes)
__ "Prival" modification *(i.e. azo-reduction method)*
__ spot test
__ other

2. Protocol
In the first trial, a plate incorporation method was employed (Ames *et al.* (1973) *Proc. Nat. Acad. Sci.* 70: 2281-2285, Ames *et al.* (1975) *Mutation Res.* 31: 347-364). For the mutant count, three plates were used, both with and without S9 mix, for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control. Each positive control also contained three plates per strain. The amount of solvent for the test substance and for the controls was 0.1 ml/plate. The doses for the first trial were routinely determined on the basis of a standard protocol: if not limited by solubility 5,000 µg or 5 µl per plate were used as the highest dose. At least five additional doses were routinely used. If less than three doses were used for assessment, at least used for assessment, at least two repeats were performed.
The results of the first experiment were then considered as a pre-test for toxicity. However, in case of a positive response or if at least three doses could be used for assessment, the first trial was included in the assessment. If the second test confirmed the results of the first, no additional repeat was performed. Doses of repeats were chosen on the basis of the results obtained in the first experiment. The independent repeat was performed as preincubation 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. For the mutant count, three plates were used for each strain and dose. An equal number of plates, filled with the solvent minus the test substance, comprised the negative control. Each positive control also contained three plates per strain. The doses of this trial were determined on the basis of the results of the plate incorporation assay. Doses are given as [µg/tube for better separation of plate incorporation and preincubation trials, despite the fact that µg/plate and µg/tube could be used synonymously.

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The toxicity of the substance was assessed in three ways. The first method was a gross appraisal of background growth on the plates for mutant determination. If a reduction in background growth was observed, it was indicated in the tables by the letter "b" after the mutant count. Where only a single "b", without any other values, is noted for a concentration, this "b" represents three plates with reduced background growth. (The same applies to the signs "c", "v", "p", "n" or"%", which may also be used in the tables). Secondly, a toxic effect of the substance was assumed when there was a marked and dose-dependent reduction in the mutant count per plate, compared to the negative controls. Thirdly, the titer was determined. Total bacterial counts were taken on two plates for each concentration studied with S9 mix. However, if an evaluation was performed only without S9 mix, the bacterial count was taken without S9 mix. The bacterial suspensions were obtained from 17-hour cultures in nutrient broth, which had been incubated at 37°C and 90 rpm. These suspensions were used for the determination of mutant counts. No standardized procedure was employed to set the bacterial suspensions at a defined density of viable cells per milliliter, since the chosen method of incubation normally produces the desired density. However, the numbers of viable cells were established in a parallel procedure by determining the titers. The dilution of bacterial suspensions used for the determination of titers was 1:1,000,000. Titers were determined under the same conditions as were the mutations, except that the histidine concentration in the soft agar was increased five-fold to permit the complete growth of bacteria. The tests were performed both with and without S9 mix. The count was made after the plates had been incubated for 48 hours at 37°C. If no immediate count was possible, plates were temporarily stored in a refrigerator.

3. Statistical Analysis: No statistical analysis was performed on the study data.

4. Evaluation Criteria:

The following criteria determined the acceptance of an assay:

a) The negative controls had to be within the expected range, as defined by published data and/ or the laboratories' own historical data; b) the positive controls had to show sufficient effects, as defined by the laboratories' experience; and c) titer determinations had to demonstrate sufficient bacterial density in the suspension. Only trials which complied with all three of the above criteria were accepted for assessment. Even if the criteria for points (b) and (c) were not met, a trial was accepted if it showed mutagenic activity of the test compound. Furthermore, an unacceptable trial would have been repeated.

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II. Results and Discussions:

Findings:
Doses up to and including 5,000 µg/plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. Evidence of mutagenic activity of BYI 08330-di-hydroxy was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed. The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant colonies compared to the corresponding negative controls.

III. Conclusions: BYI 08330-di-hydroxy was considered to be non-mutagenic without and with metabolic activation under the conditions of the Salmonella/microsome test.

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IIA 5.9 Medical and clinical data

IIA 5.9.1 Report on medical surveillance on manufacturing plant personnel

During production of BYI 08330 no accidents or incidents have occurred. Routine occupational medical surveillance of workers exposed to BYI 08330 did not reveal any unwanted effects, except two proven cases of type 4 sensitization (., 2006, M-277039-01-1).
Due to the two cases of type 4 sensitization, a survey among all potentially exposed persons did not reveal any additional cases (2005, M-257035-01-1).

IIA 5.9.2 Report on clinical cases and poisoning incidents

There are no reported clinical cases and poisoning incidents in humans
(2006, M-275046-01-1).

IIA 5.9.3 Observations on general population exposure & epidemiological studies

There are no such observations or epidemiologic studies known
(2006, M-275046-01-1).

IIA 5.9.4 Clinical signs and symptoms of poisoning and details of clinical tests

Signs or symptoms of poisoning in humans are not known. Judging from animal experiments no specific signs or symptoms are to be expected (2006, M-275046-01-1).

IIA 5.9.5 First aid measures

Remove patient from exposure/terminate exposure. Thorough skin decontamination with copious amounts of water and soap. Flushing of the eyes with lukewarm water for 15 minutes. Induction of vomiting is not required in regard of the low toxicity.

IIA 5.9.6 Therapeutic regimes

A specific antidot is not known. Therapeutic measures should consist of symptomatic and supportive treatment.

IIA 5.9.7 Expected effects & duration of poisoning as a function of exposure

Gastric lavage is not required in regard of the low toxicity of the compound. However, the application of activated charcoal and sodium sulphate may be advisable in significant ingestions.

IIA 5.9.8 Effects & duration of poisoning as a function of time

Full recovery of poisoning is to be expected, no sequelae are expected.

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IIA 5.9.9 Dermal penetration

The extent of dermal absorption of [^{14}C]-BYI 08330 was investigated *in vivo* in rats and *in vitro* on human and rat skin.

The *in vivo* study (Report No. SA 06009) was performed with a BYI 08330 OD 150 formulation exposing male rats to three dose levels at nominal concentrations of 100 µg a.i./cm^2 (10 mg/mL), 15 µg a.i./cm^2 (1.5 mg/mL), and 5 µg a.i../cm^2 (0.5 mg/mL) for periods of 1, 10, or 24 hours. Animals were sacrificed immediately thereafter. In addition, some animals were exposed for 10 hours to the three dose formulations and were sacrificed 168 hours post-dose application. A representative exposure time for farmers in the fields would be a period of 6-8 hours. In addition, residues in the skin might be absorbed over an extended period of time. Therefore, the results of the rat groups which were exposed for 10 hours and sacrificed after 168 hours have been used to derive a proposed dermal absorption value.

In vitro data are available from two studies – one performed with the SC 240 formulation (Report No. SA 05255) and another one performed with the OD 150 formulation (Report No. SA 05254) using the neat concentration (high dose: 240 or 150 mg/mL, respectively), a medium dose dilution (1.5 mg/mL) and a low dose dilution 0.05 mg/mL).

The results of the *in vivo* study performed with the OD 150 formulation (using an exposure time of 10 hours and a period of 168 hours post application) together with the results of the *in vitro* study performed with the OD 150 formulation are used to determine the dermal absorption to be used for risk assessment for the OD 150 formulation for Europe (EU).

The *in vitro* studies demonstrate that no significant difference in dermal absorption is detected between both the SC 240 and the OD 150 formulations. The same is expected for the *in vivo* results. Therefore, the results of the *in vivo* study performed with the OD 150 formulation together with the results of the *in vitro* study performed with the SC 240 formulation are used to develop a proposal for dermal absorption of BYI 08330 in the SC 240 formulation for Europe (EU).

<u>BYI 08330 OD 150 (EU):</u> Derived from the results of the studies it is proposed to use 1.19% dermal absorption to calculate systemic exposure to the neat concentration (150 mg/mL), 0.55% to calculate systemic exposure to the medium dose dilution (1.5 mg/mL) and 2.15% to calculate systemic exposure to the low dose dilution (0.05 mg/mL).

<u>BYI 08330 SC 240 (EU):</u> Derived from the results of the studies it is proposed to use 1.49% dermal absorption to calculate systemic exposure to the neat concentration (240 mg/mL), 0.57% to calculate systemic exposure to the medium dose dilution (1.5 mg/mL) and 4.15% to calculate systemic exposure to the low dose dilution (0.05 mg/mL).

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In the US, *in vitro* data are not considered to deduce *in vivo* human dermal absorption from *in vivo* rat dermal absorption. Therefore, the proposed dermal absorption for the OD 150 formulation in the US is exclusively based on the *in vivo* study.

BYI 08330 OD 150 (US): The proposed dermal absorption value for BYI 8330 is 6.2%. This is based on the total percentage absorbed after 7 days for the low dose, 10 hour exposure (5.81%) adjusted for the total recovery of 93.43%.

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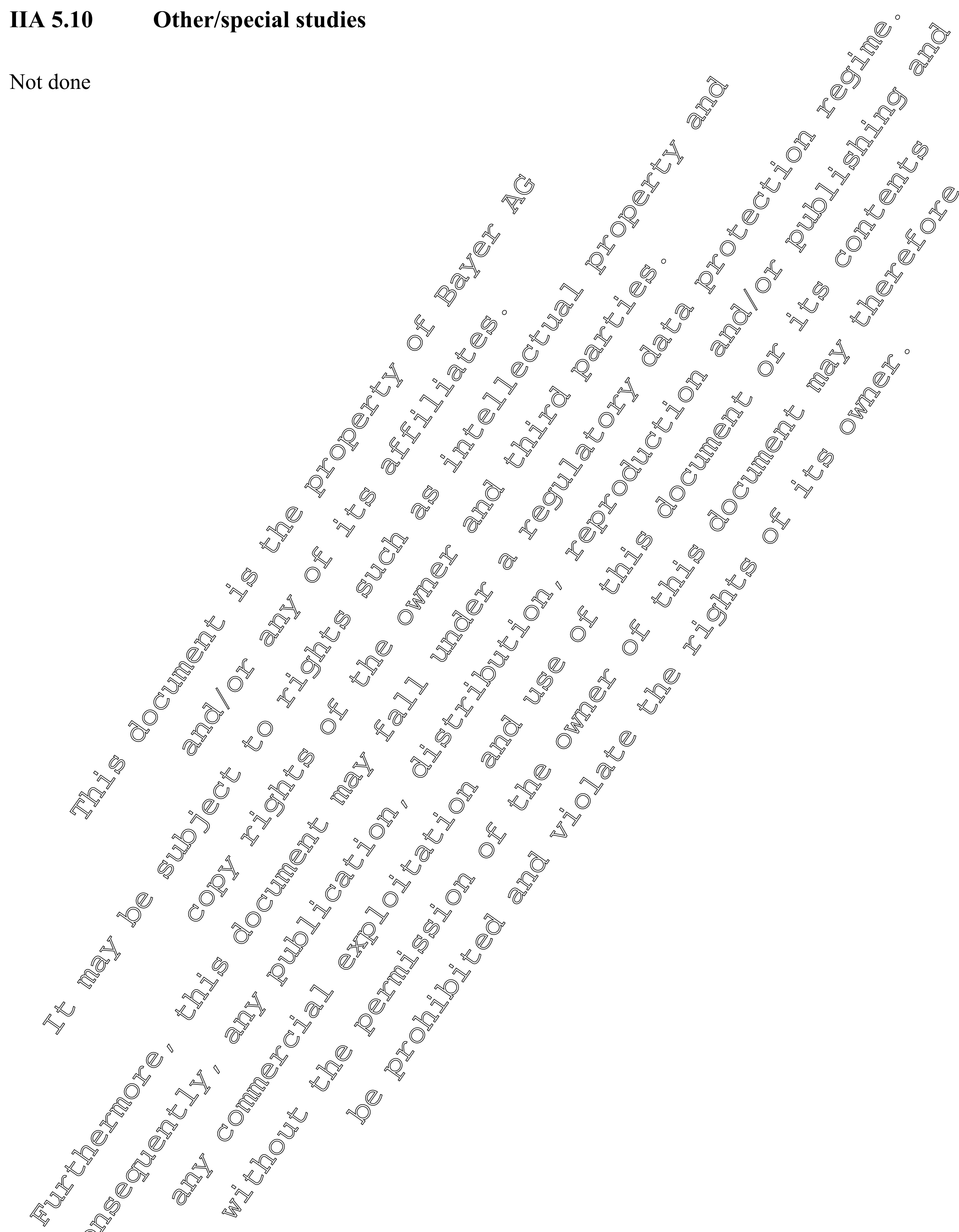
IIA 5.10 Other/special studies

Not done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IIA 5.11 Summary of mammalian toxicity and overall evaluation

BYI 08330 is an insecticidal compound and belongs to the chemical class of cyclic ketoenoles. BYI 08330 is an ACCase (Acetyl CoA Carboxylase) inhibitor. In eukaryotes and prokaryotes, ACCase is a key enzyme in fatty acid biosynthesis. The biological activity of cyclic ketoenoles correlates with inhibition of lipogenesis in treated insects, resulting in decreased lipid contents (notably triglycerides and free fatty acids), inhibition of the ability of younger insects to develop through the various growth stages, and ultimately c█inating in a diminished capacity of the insect to reproduce as adults. The insecticidal mode of action is not reflected by the results of the multiple dose toxicological studies in rodents and dogs. Rats, mice and dogs did not exhibit changes in plasma lipid parameters such as plasma triglycerides and plasma cholesterol.

BYI 08330 has a very low acute oral (LD_{50}: >5,000 mg/kg bw; females tested only), dermal (LD_{50}: >2,000 mg/kg bw) and inhalative toxicity (LC_{50}: >4,183 mg/m^3 air) in male and female rats. BYI 08330 is non-irritating to the skin although it is an irritant to the eyes and exhibits a skin sensitization potential under the conditions of the guinea pig maximization test and the Local Lymph Node Assay, whereas the Bühler test revealed no skin sensitization reactions.

BYI 08330 is considered to be non genotoxic, based on *in vitro* and *in vivo* genotoxicity studies.

A summary of the NOAELs and LOAELs from the repeated dose toxicity studies with BYI 08330 is presented in Table 5.11.1a.

In rats, subchronic treatment induced body weight declines in males at the highest dietary treatment level (10,000 ppm equal to 616 mg/kg bw/day) together with degenerative effects of the germinal epithelium of the testes resulting in abnormal spermatozoa and hypospermia in the epididymides. The effect proved to be reversible after cessation of treatment. Both genders revealed a slight alveolar macrophage accumulation in the lung at the highest dose tested.

The chronic response (12 months) in the rat was characterized by reduced terminal body weights in females, slight increases in liver weight, discoloration and/or alveolar macrophage accumulation in the lung in both genders. There were no microscopic effects on the testes and epididymides at 7,500 ppm (equal to 414 mg/kg bw/day), the highest dose tested.

The chronic response (24 months) in the rat was characterized by body weight declines, increased relative lung weights, discoloured zones in the lung (females), alveolar macrophage accumulation combined with an interstitial pneumonia in the lung, decreased absolute kidney weights with renal tubular dilatation, and slight, nonstatistical increased incidences of biliary hyperplasia/fibrosis (females only). In addition, at 7,500 ppm (equal to 373 mg/kg bw/day) typical aging changes were noted in the testes, accompanied by treatment-related subtle tubular testicular degeneration, correlating with an increased incidence of intraluminal immature/exfoliated germ cells in the epididymides.

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Unlike the rat no effects of any kind emerged in the mouse which was tested up to the limit dose in a subchronic feeding study (90 days) and in a 18-months feeding study.

BYI 08330 showed no evidence of an oncogenic potential in lifetime bioassays conducted in both the rat and the mouse.

The short- and longterm studies revealed no evidence for a neurotoxic or immunotoxic potential of BYI 08330. The acute neurotoxicity study revealed evidence of general systemic toxicity only and included decreased motor activity beginning in male rats at 200 mg/kg and clinical signs beginning at 200 mg/kg in both genders.

Subacute (28 days) percutaneous treatment with BYI 08330 in rats revealed no compound-related effects up to the limit dose of 1,000 mg/kg bw/day.

In the subacute and subchronic dog studies, body weight declines were noted at dietary levels above 2,500 ppm (equal to 72 mg/kg bw/day). Subchronic (90 days) exposure to BYI 08330 was characterized by nonadverse declines in circulating thyroid hormones at 1,200 (equal to 33 mg/kg bw/day) and 2,500 ppm. However, no changes in thyroid weight, thyroid pathology, no compensating increases in thyrotropin (TSH), or no clinical observations (e.g., neurological signs) suggestive of thyroid compromise and/or hypothyroidism were detected in either sex. Dogs showed no morphological changes in the testis up to 6,400 ppm (equal to 104 mg/kg bw/day), the highest dietary level tested.

Chronic exposure (12 months) to dogs confirmed the decline in circulating thyroid hormones at 600 and 1,800 ppm. In addition, a slight reduction in the size of the peripheral thyroid follicles was noted in two males at 1,800 ppm. However, again no changes in thyroid weight, no compensating increases in thyrotropin (TSH), or no clinical observations (e.g., neurological signs) suggestive of thyroid compromise and/or hypothyroidism were detected in either sex.
Based on the overall response of the animals to the thyroid profile that emerged over up to one year, the isolated compound-induced changes in circulating thyroid hormones observed in the dog studies are judged to be non-adverse.

No evidence for a primary reproductive toxic or teratogenic potential of BYI 08330 was determined in the multigeneration study in rats and in developmental toxicity studies in rats and rabbits, as reproductive or developmental toxicity was identified at equivalent or higher doses than parental toxicity in both species.

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Reproductive toxicity studies revealed sperm cell toxicity and infertility in male rats only after repeated high dose exposures of > 300 mg/kg bw/day (high-dose phenomenon with steep dose-response). Pharmakokinetic studies suggest the existence of active transport processes which have finite capacities for the distribution / elimination of BYI 08330-metabolites and are subject to saturation. The monovalent anionic nature of the BYI08330-enol and the BYI08330-desmethylenol at physiological pH prevents it´s passive diffusion through lipophilic cell membranes. Transporter protein assays confirmed the hypothesis that main spirotetramat metabolites may interact with organic anion transporters predominantly expressed in the membranes of proximal tubule cells of the kidneys. Thus, saturation of active transport proteins by repeated high dose exposure to spirotetramat metabolites most likely triggers a transition from first order elimination kinetics to zero-order kinetics which lead to subsequent toxicity via accumulation of these monovalent anions.

An investigative study clarified the time of onset and the origin of the BYI 08330-induced effects on the germinal epithelium. Effects appear after 10 days of high dose treatment and are due to the enol metabolite of BYI 08330. The main morphological change is a degeneration/loss of elongating spermatids (step 9 to 14) together with a degeneration of round spermatids (step 7 to 8). Testicular/sperm cell toxicity in rats was always accompanied by systemic toxicity. Reversibility of testicular toxicity was proven in a 90-day rat study with a 4-week recovery group. There was no evidence for a hormonal dysregulation. Furthermore, the testicular effects occurred in one species only. In vitro tests using hepatocytes from male rats, mice and humans revealed species differences in the glucuronidation of the BYI 08330-enol. Unlike the rat, mouse and human hepatocytes are able to conjugate the BYI 08330-enol. The glucuronidation reduces the systemic enol burden which has been proven to be the toxophore responsible for the degenerative testicular effects and provides alternative elimination pathways by enabling the utilization of separate active transport mechanisms in the kidney and possibly in the liver (across the canalicular membrane into the bile) rendering these species less susceptible. A separate position paper summarizes (M-297775-01-1) the high dose reproductive effects in male rats and their relevance to humans.

Thus, the effects of BYI 08330 on rat sperm have been characterized from a toxicological and metabolic/toxicokinetic perspective, and provide support for the conclusion that the development of non-linear elimination kinetics are high-dose phenomena, with an increased sensitivity of the rat, and would not be encountered by the human even under the most extreme levels of anticipated prolonged and repeated exposure. Therefore, BYI 08330 is not considered to represent a reproductive hazard to humans at the expected low dose exposure scenarios routinely generated through the agricultural use of the chemical.

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Table 5.11.1a: Summary of NOAELs and effects at the LOAEL in repeat dose toxicity studies with BYI 08330

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Species	Study (dosage)	NOAEL		Effects at LOAEL (and other comments)
		[ppm]	[mg/kg bw/day]	
Rat	Oral, feeding, 28 days (females only) (0 – 500 - 5,000 ppm)	5,000 (f)	502 (f)	Females: none
	Dermal, 28 days (0 – 100 – 300 - 1,000 mg/kg bw)		systemic: 1,000 local: 1,000	systemic: none local: none
	Oral, feeding, 90 days (0 – 150 – 600 - 2,500 – 10,000 ppm)	2,500 (m) 2,500 (f)	148 (m) 188 (f)	Males: reduced body weight gain, reduced terminal body weight, abnormal epididymal spermatozoa and hypospermia, testicular tubular degeneration, increased incidences of accumulation of alveolar macrophages Females: increased incidences of accumulation of alveolar macrophages
	Chronic feeding (12 mths) (0 – 250 - 3,500 - 7,500♂ / 12,000♀ ppm)	250 (m) 3,500 (f)	13.2 (m) 255 (f)	Males: increased incidence of accumulation of alveolar macrophages Females: decreased terminal body weight, yellow and brown staining in the perigenital area and tail, increased relative liver weight, discoloration of the lung and increased incidence of accumulation of alveolar macrophages
	Oncogenicity feeding (24 mths) (0 – 250 - 3,500 - 7,500♂ / 12,000♀ ppm)	250	12.5 (m) 16.8 (f)	Males: decreased absolute kidney weights, increased incidence of renal tubular dilatation Females: decreased absolute kidney weights, increased incidence of renal tubular dilatation Not shown to be oncogenic.

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Rat	2-Generation study (0 – 250 - 1,000 - 6,000 ppm)	parental: 1,000 reproduct: 1,000 (m) 6,000 (f) neonatal: 1,000	70.7 (m) 82.5 (f) 79.5 (m) 484.7 (f)	parental (m): body weight gain declines (P° and F1 gen), decreased terminal body weights and, increased renal multifocal tubular dilatation (F1) parental: (f): reduced food consumption during lactation (P and F1 gen), reduced body weights (end premating), body weight gain declines (premating), decreased terminal body weights and increased multifocal renal tubular dilatation (F1) reproductive (m): abnormal sperm cell morphology (F1) reproductive (f): none neonatal: decreased body weight on lactation day 21 (F1) and lactation day 14 and 21 (F2)
	Developmental toxicity (0-20-140-1,000 mg/kg)		maternal: 140 developm: 140	maternal: reduced feed intake, transient body weight loss, reduced terminal body weight, reduced corrected body weight gain developmental: marginal reduction of placental weight, distinctly decreased fetal weights, slightly increased incidence of common unspecific malformations, increased incidence of skeletal variations (wavy ribs, 14th ribs, combined osseous and cartilaginous findings), and increased incidence of retarded ossification
	Developmental toxicity (0-10-35-140 mg/kg)		maternal: 140 developm: 140	maternal: none developmental: none
Rabbit	Developmental toxicity (0-10-40-160 mg/kg)		maternal: 10 developm: 40	maternal: abortion in one animal developmental: increased incidence of distinct liver lobulation
Mouse	Oral, feeding, 28 days (males only) (0 – 500 - 5,000 ppm)	5,000	1,415 (m)	Males: none
	Oral, feeding, 90 days (0 – 70 – 350 - 1,700 - 7,000 ppm)	7,000	1,305 (m) 1,515 (f)	Males: none Females: none
	Oncogenicity feeding, 78 weeks (0 – 70 - 1,700 - 7,000*/ 6,000 ppm) *Dose reduced to 6,000 ppm after week 12	7,000 / 6,000	1,022 (m) 1,319 (f)	Males: none Females: none Not shown to be oncogenic

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Dog	Oral, feeding, 28 days (0 – 100 – 400 - 1,600 – 6,400 ppm)	1,600	42 (m) 70 (f)	Males: decreased body weight and food consumption Females: decreased body weight and food consumption
	Oral, feeding, 90 days (0 – 150 – 300 - 1,200 - and 4,000*/2,500 ppm) *Dose reduced to 2,500 ppm after week 2	2,500	81 (m) 72 (f)	Males: body weight decline (week 1 and 2) Females: body weight decline (week 1 and week 2)
	Oral, feeding, 1 year (0 – 200 – 600 - 1,800 ppm)	600 (m) 1,800 (f)	20 (m) 48 (f)	Males: reduced size of peripheral thyroid follicles in 2/4 animals Females: none

m males; f females

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Acceptable daily intake (ADI)

Based on the comprehensive toxicological data base, no effects of particular concern for consumer risk assessment have been identified. There is no indication of a genotoxic, a functional immunotoxic, a neurotoxic, an endocrine, an oncogenic, a teratogenic or a primary reproductive toxic potential of BYI 08330.

Table 5.11.1b: NOAELs and LOAELs for derivation of the proposed ADI

Data point	Study type	NOAEL	LOAEL
5.3.4	Oral 1-year, dog	M: 600 ppm (20 mg/kg bw/day) F: 1,800 ppm (48 mg/kg bw/day)	M: 1,800 ppm (55 mg/kg bw/day) F: None
5.5.1	Oral 1-year, rat	M: 250 ppm (13.2 mg/kg bw/day) F: 3,500 ppm (255 mg/kg bw/day)	M: 3,500 ppm (189 mg/kg bw/day) F: 12,000 (890 mg/kg bw/day)
5.5.2	Oral 2-year, rat	M: 250 ppm (12.5 mg/kg bw/day) F: 250 ppm (16.8 mg/kg bw/day)	M: 3,500 ppm (169 mg/kg bw/day) F: 3,500 (229 mg/kg bw/day)
5.5.3	Oral 1.5-year, mouse	7,000/6,000 ppm M: 1,022 mg/kg bw/day F: 1,319 mg/kg bw/day	None
5.6.1/02	Two-generation reproduction, rat	1,000 ppm (parental) M: 70.7 mg/kg bw/day F: 82.5 mg/kg bw/day	6,000 ppm M 419.3 mg/kg bw/day F: 484.7 mg/kg bw/day
5.6.11/02	Rabbit teratology	10 mg/kg bw/day	40 mg/kg bw/day
5.6.10/02+03	Rat teratology	140 mg/kg bw/day	1,000 mg/kg bw/day

It is therefore appropriate to set the ADI based on the long-term NOAEL of the most sensitive species (rat), i.e. the NOAEL of 13.2 mg/kg bw/day (males) in the repeated dose 1-year oral toxicity study in rats. Since the carcinogenicity study in rats is designed to address the oncogenic potential of the chemical and thus is incomplete as a chronic study, the NOAEL of the carcinogenicity study has not been used for dietary risk assessment.

An ADI for BYI 08330 of 0.132 mg/kg bw/day bw is proposed based on applying a 100-fold uncertainty factor to the NOAEL of 13.2 mg/kg bw/day.

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Acute reference dose (ARfD)

BYI 08330 was of very low acute oral toxicity in rats. This was demonstrated by the absence of any clinical signs at the limit dose of 2,000 mg/kg bw in the standard acute oral toxicity study. However, the basis for establishing an ARfD should be a single dose experiment with an extended study protocol.

For this purpose, the acute neurotoxicity screening study in the rat, involving a variety of very specific neurobehavioral investigations, is considered appropriate. This study established a NOAEL of 100 mg/kg bw/day, based on urine stains and slight motor activity declines in male rats at 200 mg/kg bw/day.

An acute reference dose for BYI 08330 of 1 mg/kg bw/day is proposed based on applying a 100-fold uncertainty factor to the NOAEL of 100 mg/kg bw/day.

Long-term systemic AOEL

A long-term systemic AOEL for BYI 08330 is not considered appropriate because of the short duration of use of this product.

Short-term systemic AOEL

Occupational exposure to BYI 08330 is expected to be intermittent over a two to three weeks duration. Based on the projected use pattern, toxicity studies of 13 weeks duration are most relevant for assessing occupational exposure. Short term studies applicable for operator exposure risk assessment are listed in the following table.

Table 5.11.1c: NOAELs and LOAELs for derivation of the proposed AOEL

Data point	Study type	NOAEL	LOAEL
5.3.2/01	Oral 90-day, rat	2,500 ppm M: 148 mg/kg bw/day F: 188 mg/kg bw/day	10,000 ppm M: 616 mg/kg bw/day F: 752 mg/kg bw/day
5.3.2/02	Oral 90-day, mouse	7,000 ppm M: 1305 mg/kg bw/day F: 1515 mg/kg bw/day	None
5.3.3	Oral 90-day, dog	2,500 ppm M: 81 mg/kg bw/day F: 72 mg/kg bw/day	4,000 ppm (week 1-2) mg/kg bw/day not calculated
5.6.11/02	Rabbit teratology	10 mg/kg bw/day	40 mg/kg bw/day
5.6.10/02+03	Rat teratology	140 mg/kg bw/day	1,000 mg/kg bw/day

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The NOAELs of the 90-day oral toxicity studies are clearly higher than the NOAEL of 10 mg/kg bw/day observed in the rabbit teratology study. The NOAEL derived from the rabbit teratology study is based on abortion in one female rabbit at 40 mg/kg bw/day, secondary to maternal toxicity. Even at the next higher dose of 140 mg/kg bw/day, abortions occurred only as a consequence of maternal toxicity, with no indications of chemical specific effects in offsprings exposed in utero. Therefore, the selection of the NOAEL of 10 mg/kg bw/day derived from the rabbit teratology study is very conservative. Toxicokinetic studies indicate that BYI 08330 is gastrointestinally absorbed with approximately 95-96% of the applied dose (2 mg/kg bw). No adjustment for oral absorption is required.

A short-term systemic AOEL for BYI 08330 of 0.1 mg/kg bw/day is proposed based on applying a 100-fold uncertainty factor to the NOAEL of 10 mg/kg bw/day in the teratology study in rabbits, and applying a 100% gastrointestinal absorption derived from the rat metabolism study.

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