UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

Date: March 12, 2014

SUBJECT: **Flupyradifurone**: Review of toxicology studies for Section 3 registration.

PC Code: 122304
Decision No.: 471642
Petition No.: NA
Risk Assessment Type: NA
TXR No.: 0056910
MRID No.: See table below.

DP Barcode: D415670
Registration No.: 264-RRUG
Regulatory Action: Section 3 Registration
Case No.:
CAS No.: 951659-40-8
40 CFR: TBD

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The registrant, Bayer CropScience, submitted a series of toxicology studies on flupyradifurone for Section 3 registration in the US. These studies have gone through a global review process; Australian toxicologist is the primary reviewer; PMRA and US EPA are secondary reviewers. The data evaluation reports (DERs) are attached; these DERs represent the final reviews reflecting the HED/OPP guideline for evaluating the toxicity data. The following table presents the MRIDs and citations of the evaluated studies.

MRID	Citation and S tudy Type
48844109	Kubaszky, R. (2012) Metabolite(difluoroethyl aminofuranone):14 day oral toxicity:
48844110	Kubaszky, R. (2012) Metabolite (difluorethyl aminofuranone): 28-day oral toxicity .
48844111	Krebber, R. (2009) 90-Day Toxicity Study -rat
48844112	Krebber, R. (2009) 90-Day Toxicity Study-mouse
48844113	Kennel, P. (2012) Metabolite (Difluoroacetic acid): 90-Day toxicity study -rat

MRID	Citation and Study Type
48844114	Eigenberg, D. (2010) 90-Day Toxicity Feeding Study-dog
48844115	Cada, A. (2012) 28-Day dermal toxicity study- rat
48844116	Langrand-Lerche, C. (2010) – Development toxicity study-rat
48844117	Kennel, P. (2012) Developmental Toxicity Study-rabbit
48844119	Milius, A. (2011) Two-generation reproduction study-rat
48844120	Milius, A. (2010) Doser-range-finding study (for reproduction study-MRID 48844119)
48844121	Cada, A. (2012) 1-Year feeding study-dog
48844122	Kennel, P. (2012) Carcinogenicity Study-mouse
48844123	Garcin, J. (2012) Chronic/Carcinogenicity study-rat
48844124	Herbold, B. (2009) Salmonella/Microsome Test-bacteria
48844125	Sokolowski, A. (2011) Salmonella typhimurium Reverse Mutation-bacteria
48844126	Sokolowski, A. (2010) Salmonella typhimurium Reverse Mutation bacteria
48844127	Sokolowski, A. (2011) Salmonella typhimurium Reverse Mutation bacteria
48844128	Entian, G. (2009) V79/HPRT-Test in vitro –forward mutation.
48844129	Hall, C. (2010) Metabolite (BCS-AA56716; DFA) –Gene mutation assay (V79/HPRT)
48844130	Wollny, H. (2011) Metabolite (difluoroethyl-amino-furanone) gene mutation assay (V79/HPRT)
48844131	Thum, M. (2009) In vitro Chromosome Aberration Test (CH V79 cells)
48844132	Hall, C. (2010) Metabolite (BCS-AA56716; DFA) chromosomal aberration assay in CH-V79 cells)
48844133	Hall, C. (2011) Metabolite (difluoroethyl-amino-furanone) chromosomal aberration assay in CH-V79 cells)
48844134	Herbold, B. (2009) Micronucleus-Test on the male mice
48844135	Wieland, J. (2011) Micronucleus Assay in Bone Marrow Cells- mouse
48844136	Hall, C. (2011) Micronucleus Assay in Bone Marrow Cells-mouse
48844137	Hall, C. (2011) In vivo Unscheduled DNA Synthesis in Rat Hepatocytes
48844138	Garcin, J. (2011) An Acute Neurotoxicity Study -rat
48844139	Garcin, J. (2011) A 90-Day Neurotoxicity Study-rat
48844140	Gilmore, R. (2012) A Developmental Neurotoxicity Study-rat
48844141	Klempner, A. (2012) [Pyridinylm ethyl-^{14}C] –ADME study-rat
48844142	Koester, J.; Weber, E. (2011) Quantitative Whole Body Autoradiography-rat
48844143	Weber, E. (2011) [Furanone-4-^{14}C]- ADME
48844144	Koester, J. (2011) Quantitative Whole Body Autoradiography
48844145	Koester, J. (2012) [Furanone-4-^{14}C)] – Metabolism in organs and tissues
48844146	Weber, E. (2011) [Ethyl-1-^{14}C] ADME
48844147	Koester, J. (2011) [Ethyl-1-^{14}C)]- Metabolism in organs and tissues
48844148	Repetto, M. (2011) 28-Day Immunotoxicity Study -rat
48844149	Capt, A. (2009) Exploratory 28-Day Toxicity Stud (gavage)-rat
48844150	Blanck, M. (2008) Exploratory 28-Day Toxicity (dietary)-rat
48844151	Blanck, O. (2007) Preliminary 28-Day Toxicity Study-mouse
48844152	Odin-Feurtet, M. (2008) BYI 02960: Preliminary 28-Day Toxic
48844153	Kennel, P. (2011) Metabolite (BCS-AA56716; DFA) Preliminary 14-day toxicity-rat
48844557	Odin-Feurtet, M. (2010) BYI 02960 (SL200) - In vivo Dermal absorption-rat
48844558	Odin-Feurtet, M. (2010) BYI 02960 (SL200): Comparative dermal absorption in human & rat skin.
44988432	Mochizuki, N.; Kanaguchi, Y. (1997) Metabolite (IM-0)--Reverse Mutation-bacteria
44988502	Mochizuki, N.; Kanaguchi, Y. (1994) Metabolite (IC-0)--Reverse Mutation -bacteria
49113601	Kennel, P. (2013) Range-Finding Study for Developmental rabbit study (MRID 48844117)

Primary review: Australia
Secondary review: US EPA

14-day oral toxicity study in the rat

Report: Kubaszky R (2012) BYI 02960-difluoroethyl aminofuranone: A 14-day dose range finding toxicity/palatability study in rats. CiToxLab Hungary Ltd., H-8200 Veszprem, Szabadsagpuszta, Hungary. Report No.: 11/116-100PE. February 24, 1012. MRID 48844109. Sponsor (Bayer CropScience AG) ID No.: M-426158-01-2. Unpublished.

Guidelines: Not applicable (only preliminary and explorative study design)
GLP: Study not performed under GLP, but laboratory GLP-certified

Executive summary

In a 14-day dose-range finding study (MRID 48844109), BYI 02960 metabolite, difluoroethyl aminofuranone (batch number NLL 8671-12-1: a white powder, 98.9 % w/w purity), was administered in the diet to groups of Wistar rats (5/sex/group) for at least 14 days at concentrations of 0, 1280, 3200, 8000 and 20000 ppm (males: 0, 35, 339, 736 and 1226 mg/kg body weight/day. Females: 0, 135, 335, 741 and 2254 mg/kg body weight/day). Test animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight was recorded on days, -7, 0, 1, 3, 6, 7, 10, 13 and 14. Food consumptions were determined on days 0, 1, 3, 6, 7 ,10 and 13. Hematology and clinical chemistry parameters were determined at the end of the study. All test animals were necropsied, selected organs weighed, and a range of tissues were retained in fixative. No histopathological examination was performed.

There was no unscheduled mortality during the study and no clinical signs during the 14-day in test animals. Adverse effects considered related to BYI 02960-difluoroethyl aminofuranone administration under the conditions of this study were noted on the animal body weight and/or body weight gain values in the 3200, 8000 and/or 20000 ppm male and/or female animals, with an apparent dose response.

At 3200 ppm, statistically lower body weight gain was noted in males between Days 0 and 1 (1.4 g *vs.* 7.4 g) as well as when calculated for the whole duration of the study (Days 0 to 14, 78 g *vs.* 94.6 g, $p < 0.05$, 37.04% *vs.* 45.67 %). In the Low-mid dose 3200 ppm Group 3 females, statistically lower body weights up to -11.93% were recorded approximately after 1 week of treatment on Day 6 and towards the end of the treatment on Days 10, 13 and 14.

At 8000 ppm, statistically significant decrease in mean body weights were noted in the Group 4 males (up to ↓16.48%) after Day 3 of the treatment in the females (up to↓15.88%,), after Day 6, until its completion on Day 14. In the 20000 ppm Group 5 animals, body weight loss was noted in both males (↓32%) and females (↓30%) between Days 0 and 14.

The mean daily food consumption evaluated based on the cage-based weekly food consumption of male and female rats was lower than control in the 8000 and 20000 ppm Groups 4 and 5, up to - 59% lower in the males and up to - 25% in the female at 20000 ppm, effects considered possibly associated with test item administration. However, spillage was noted in the High dose Group 5 females and the dose response was not clear.

Statistically lower than control reticulocyte mean values were noted in the 20000 ppm males and 8000 and 20000 ppm females and were regarded as possibly associated with BYI 02960-difluoroethyl aminofuranone administration.

Lower than control glucose blood levels were noted in both males and females in all the dose groups, attaining statistical significance at above and including 3200 ppm in the males, with mean values up to - 49% lower than control, and at all the dose levels tested in the females, with mean values of up to - 52%, $p < 0.01$. Cholesterol mean values increased in the 3200 ppm group and above to up to 65% in the males, and up to 49% in the females. Albumin and A/G ratio were slightly higher than control, attaining statistical significance at 8000 and/or 20000 ppm in the males and in both males and females, respectively.

In addition, higher urea was noted in the 20000 ppm males, and in the 20000, and 8000 ppm females. Potentially test item-related macroscopic changes were observed at necropsy in the High dose group fed diet with 20000 ppm BYI 02960-difluoroethyl aminofuranone. These consisted of small prostate and seminal vesicles in 5/5 males and small spleen, in 2/5 males, were associated with the potential test item related changes in the terminal body weights and correlated with the organ weight changes, however, a definitive attribution cannot be made without histopathological evaluation.

In conclusion, based on the effects noted in the current 14-day preliminary study and in correlation with the previous data available at the Sponsor, the dose levels selected were 200, 800 and 3000 ppm (mg/kg diet). These dose levels were considered to be suitable for an upcoming 28-day dietary study with BYI 02960-difluoroethyl aminofuranone.

I. Materials and Methods

A. Material

1. Test Material:	BCS-CC98193, BYI 02960-difluoroethyl aminofuranone
Description:	White powder
Lot/Batch:	NLL 8671-12-1
Purity:	98.9%
CAS:	1134834-71-1
Stability of test compound:	Stable from 500 to 20000 ppm for at least 7 weeks in the diet storage room (approximately 15 - 21 °C) and at least 14 days in the animal room (approximately 22 ± 3 °C)

2. Vehicle and /or positive control: None

3. Test animals:

Species:	Rat
Strain:	Crl:WI rats
Age:	7 weeks
Weight at dosing:	196 to 227 g for the males; 148 to 174 g for the females
Source:	Charles River Laboratories, Germany
Acclimation period:	2 weeks
Diet:	Ssniff® SM R/M-Z+H "Autoclaved Complete Feed for rats and mice - Breeding and maintenance" (by Ssniff Spezialdiäten GmbH, D-59494 Soest Germany), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were group-housed (5 animals/sex/cage).

Environmental conditions:	Temperature:	20.1 - 24.1 °C
	Humidity:	31 - 68%
	Air changes:	15 to 20 changes per hour
	Photoperiod:	Alternating 12-hour light and dark cycles (6 am - 6 pm)

B. Study Design

1. In life dates

15 to 29 September 2011 at CiToxLAB Hungary Ltd. H-8200 Veszprém, Szabadságpuszta - Hungary.

2. Animal assignment and treatment

There were 5 rats per sex per dose group. Animals were assigned to dose groups using a randomization by weight. BYI 02960-difluoroethyl aminofuranone was administered in the diet for 14 days to Wistar rats at the following doses: 0, 1280, 3200, 8000 and 20000 ppm (equating to approximately 135, 339, 736 and

1226 mg/kg/day in males and 135, 335, 741 and 2254 mg/kg/day in females). A negative control group received plain diet.

Table 5.8-23: Study design

Test Group	Diet Concentration (ppm)	Animals assigned	
		Male	Female
1/Control	0	5	5
2/Low dose	1280	5	5
3/Low mid dose	3200	5	5
4/High mid dose	8000	5	5
5/High dose	20000	5	5

3. Diet preparation and analysis

BYI 02960-difluoroethyl aminofuranone was incorporated into Ssniff® SM R/M-Z+H "Autoclavable Complete Feed for Rats and Mice - Breeding and Maintenance" by Ssniff Spezialdiäten GmbH, D-59494 Soest Germany and mixed for up to approximately 14 minutes (approximately 6 minutes for premix preparation, and 4-8 minutes for preparation of the complete diets), in a room where the temperature and humidity were controlled. Following mixing, pellets were prepared by simple compression; no binding agents, steam, external heat, any other process or substance were used that might affect BYI 02960-difluoroethyl aminofuranone or the quality of the diets. Similar diet preparation procedures were used to generate control diet (0 mg BYI 02960-difluoroethyl aminofuranone/kg diet).
The prepared diets were stored at room temperature under dry conditions, in sewed bags pending and during transport to CiToxLAB Hungary Ltd. At CiToxLAB Hungary Ltd., the prepared diets were stored in areas designated for diet storage at room temperature (approximately 15 - 21 °C), under dry conditions, pending transfer to animal room at approximately 22 ± 3 °C for animal feeding.

Analyses of the diets for homogeneity and/or concentration of BYI 02960-difluoroethyl aminofuranone were performed based on an analytical method validated at CiToxLAB Hungary Ltd. (11/116-316AN). Concentration and homogeneity assessment were performed at the diet arrival; additional concentration measurements were conducted at the end of the study, from the remaining diet collected from the animal room on Day 13. At receipt of the diets five samples were taken from different areas (top, middle and bottom) of the diet containers. On Day 13 five samples were collected from the remaining diet in the animal room, from different areas (top, middle and bottom) of the hoppers. One sample was taken from the control. At receipt of the diets the samples were homogenized and three replicate extractions were carried out from each sample in order to test the homogeneity. On Day 13 only the concentration of the diet was verified. The samples were mixed and five replicate extractions were carried out.

Diet samples were stored at room temperature, dry, pending analysis on the same day. No test item was detected in the Control samples. The test item was homogenously distributed in the diet. The concentration of the test item in the diet samples varied between 91% and 102% of the nominal values,

within the acceptable range of 100 ± 10% and thus these results were considered suitable for the study purposes.

4. Statistics

The heterogeneity of variance between groups was checked by Bartlett's homogeneity of variance test. Where no significant heterogeneity was detected, a one-way analysis of variance was carried out. If the obtained result was positive, Duncan's Multiple Range test was used to assess the significance of inter-group differences. Where significant heterogeneity was found, the normal distribution of data was evaluated by Kolmogorov-Smirnov test.

In case of abnormal distribution, the non-parametric method of Kruskal-Wallis One-Way analysis of variance was applied. If the result was positive, the inter-group comparisons were performed using Mann-Whitney U-test. The mean and standard deviations values, the frequency of clinical observations and macroscopic findings were calculated as applicable.

B. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily. All animals were observed for clinical signs at least once daily at approximately the same time, with minor variations as practical. Detailed physical examinations were performed on all animals outside the home cage at randomization (Day -7), on the first day of treatment and at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight

Body weights were recorded with a precision of 1 g at randomization (Day - 7), on the first day of treatment (Day 0, prior to start of treatment), then on Days 1, 3, 6, 7, 10, and 13 and 14 (fasted, to allow calculation of the relative organ weights).

3. Food consumption and compound intake

Food consumption were measured pre-treatment from Day - 7 to Day 0. The remaining, non-consumed food given as of Day 0 was weighed with a precision of 1g on Days 1, 3, 6, 7, 10, and 13 during the treatment period.

4. Clinical pathology

<u>Blood sampling</u>

Blood samples for clinical pathology evaluation were collected from all animals immediately prior to the scheduled necropsy on Day 14, after an overnight period of food deprivation of animals, by heart puncture under pentobarbital anaesthesia.

Three samples were taken from each animal, one for haematology (approximately 1.2 mL blood into K_3-EDTA tubes, 1.6 mg/mL blood), one for determination of blood clotting times (approximately 1.2 mL

blood for APTT and PT measurements, into sodium citrate tubes) and the third one to obtain serum samples (up to 2.4 mL blood as practical, into tubes with no anticoagulant) for clinical chemistry.

Hematology

Red blood cell count, haemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cell volume, reticulocyte count, white blood cell count and differential count evaluation and platelet count and mean platelet volume were assayed using an Advia 120.

Blood smears were prepared for all animals but their examination was not considered required under the conditions of this preliminary study.

Prothrombin time and Activated partial thromboplastin time were assayed on an AMAX Destiny Plus Coagulometer.

Clinical chemistry

Total bilirubin, glucose, urea, creatinine, total protein, albumin, total cholesterol, triglycerides, phosphorus, sodium, potassium, calcium and chloride concentrations, and γ glutamyltransferase, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed using an VITROS equipment. Bile acids were evaluated on a Lory 2000.

5. Sacrifice and pathology

Necropsy and macroscopic examination were performed on all animals, which survived to the scheduled termination on Day 14, after the blood collection for clinical pathology evaluation. The animals were euthanized by exsanguination under pentobarbital anaesthesia.

After exsanguination the external appearance were examined, cranium, thoracic and abdominal cavities were opened and the appearance of the tissues and organs was observed macroscopically. Any abnormalities were recorded with details of the location, color, shape and size, as appropriate.

The following organs were weighed: Adrenal glands, brain, epipidymides, heart, kidney, liver, prostate, ovaries, seminal vesicles with coagulating glands, spleen, testes, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix). Paired organs were weighed together.

All the organs weighed as above and any organs with macroscopic abnormalities were retained in fixative as applicable (testes and epididymides in Bouin’s solution, all other organs in 10% buffered formalin solution). No histopathology evaluation was performed during the study in agreement with the Sponsor, in consultation with the Study Director.

II. Results and discussion

A. Observations

1. Clinical signs of toxicity

There were no clinical signs observed during the study.

2 .Mortality

There was no unscheduled mortality during the study.

B. Body weight and body weight gain

At 20000 ppm, body weight loss was noted in both males and females between Days 0 and 14, with lower values of up to -104.02% in the males (- 3.8 g *vs.* 94.6 g, - 1.88% *vs.* 45.67%) and up to -122.52% in the females (-10 g *vs.* 44.4 g, - 6.48% *vs.* 27.10%).

At 8000 ppm, statistically lower mean body weights up to -16.48% were noted in the males after Day 3 of the treatment, and up to -15.88% in the females, after Day 6, until its completion on Day 14. The body weight gains between Days 0 and 14 were up to -54% lower than control in the males (43.4 g *vs.* 94.6 g, 20.76% *vs.* 45.67%) and up to -64% in the females (16 g *vs.* 44.4 g, 9.93% *vs.* 27.10%).

At 3200 ppm, statistically lower body weight gain was noted in males between Days 0 and 1 (1.4 g *vs.* 7.4 g) as well as when calculated for the whole duration of the study (Days 0 to 14, 78 g *vs.* 94.6 g, 37.04% *vs.* 45.67 %). In females, statistically lower body weights up to -11.93% were recorded approximately after 1 week of treatment on Day 6 and towards the end of the treatment on Days 10, 13 and 14. The body weight gain was also lower than control between Days 3 and 6 (4.2 g *vs.* 13.4 g) as well as when calculated for the whole duration of the study (Days 0 to 14, 26.2 g *vs.* 44.4 g, 16.76% *vs.* 27.10 %).

There were no body weight or body weight gain changes considered related to test item administration, or to reflect an adverse effect, at 1280 ppm BYI 02960-difluoroethyl aminofuranone.

Table 5.8-24: Summary of body weight and body weight gain data (g)

Dose levels of Test item (ppm)	0	1280	3200	8000	20000
Male					
Initial BW (Day 0) (%C)	207.6	211.0(102%)	210.6(101%)	209.0(101%)	208.0(100%)
BW (Day 1) (%C)	215.0	218.8 (102%)	212.0 (99%)	202.2 (94%)	198.0* (92%)
BW (Day 7) (%C)	265.0	266.0 (100%)	255.6 (96%)	235.2** (89%)	196.2** (74%)
Final BW (Day 14) (%C)	302.2	301.2 (100%)	288.6 (95%)	252.4** (84%)	204.2** (68%)
BWG (Day 0-1) (%C)	7.4	7.8 (105%)	1.4* (19%)	- 6.8** (-192%)	- 10.0** (-235%)
BWG (Day 1-3) (%C)	17.6	15.4 (87%)	16.2 (92%)	6.8** (39%)	-9.4** (-53%)
BWG (Day 7-10) (%C)	30.2	26.2 (87%)	25.4 (84%)	16.0** (53%)	11.2** (37%)
Overall BWG (Days 0-14) (%C)	94.6	90.2 (95%)	78.0 (82%)	43.4** (46%)	- 3.8** (-104%)
Female					
Initial BW (Day 0)(%C)	163.4	160.2 (98%)	156.8 (96%)	158.8 (97%)	155.4 (95%)
BW (Day 1) (%C)	163.0	164.2 (101%)	157.0 (96%)	153.8 (94%)	143.8** (88%)
BW (Day 7) (%C)	187.8	186.6 (99%)	174.0 (93%)	168.6* (90%)	147.4** (78%)
Final BW (Day 14) (%C)	207.8	200.4 (96%)	183.0** (88%)	174.8** (84%)	145.4** (70%)
BWG (Day 0-1) (%C)	-0.4	4.0 (900%)	0.2 (150%)	- 5.0 (-1350%)	- 11.6** (3000%)
BWG (Day 1-3) (%C)	10.0	8.2 (82%)	8.0 (80%)	6.4 (64%)	- 2.6** (-126%)
BWG (Day 7-10) (%C)	18.2	10.6* (58%)	11.2 (62%)	13.4 (74%)	3.6** (20%)
Overall BWG (Days 0-14) (%C)	44.4	40.2 (90%)	26.2** (59%)	16.0** (36%)	- 10.0** (-123%)

* : p <0.05 ** : p <0.01 Data excerpted from pages

C. Food consumption and compound intake

The mean daily food consumption evaluated based on the cage-based weekly food consumption of male and female rats was lower than control in the 8000 and 20000 ppm Groups 4 and 5, up to - 59% lower in the males and up to - 25% in the female at 20000 ppm, effects considered possibly associated with test item administration. However, spillage was noted in the High dose Group 5 females, making assessment difficult.

Possible test-item related adverse effects were noted on the food conversion efficiency FCE values (g/g, calculated as weekly body weight gain g/weekly food consumption g) at 3200, 8000 and 20000 ppm, up to - 16.45%, - 30.26% and - 85.09% in the Groups 3, 4 and 5 males and - 18.84%, - 22.11% and - 85.09% in the Groups 3, 4 and 5 females, respectively.

The mean intake over the 14 days was 135, 339, 736 and 1226 mg/kg bw/day for the males and 135, 335, 741 and 2254 mg/kg bw/day for the females from Groups 2, 3, 4 and 5, respectively.

Table 5.8-25: Summary of food consumption (g)

Dose levels of Test item (ppm)	0	1280	3200	8000	20000
Male					
FC (Days 0-7) (%C)	25.4	26.6 (105%)	26.0 (102%)	20.0 (79%)	10.5 (41%)
FC (Days 7-13) (%C)	28.8	29.5 (102%)	28.4 (99%)	24.5 (85%)	15.9 (55%)
Female					
FC (Days 0-7) (%C)	19.1	18.8 (98%)	17.7 (93%)	15.0 (79%)	18.5 (97%)
FC (Days 7-13) (%C)	22.7	20.2 (89%)	19.4 (85%)	17.7 (78%)	17.1(75%)

D. Clinical pathology

1. Hematology

Statistically lower than control reticulocyte mean values were noted in the 20000 ppm males (3.04% *vs.* 5%) and 8000 and 20000 ppm females (3.16% and 2.28%, *vs.* 3.94% in the control group) and were regarded as possibly associated with BYI 02960-difluoroethyl aminofuranone administration, although the values in the test item treated groups remained only slightly lower than the physiological ranges. The white blood cell count was statistically lower than control at all the dose levels tested in the male animals and lower than concurrent historical control mean values; however, the mean values were comparable with the values recorded in the females, including control, there were no similar statistical differences in the females and thus a test item related effect was regarded as equivocal.

Other parameters showed on occasion statistically significant variations, including slightly higher red blood cell count RBC or haemoglobin HGB in the 8000 ppm and 20000 ppm males but not in the females, or lower PLT in the 3200 ppm males only. In the absence of a clear dose or gender response and/or as the values were generally comparable with the physiological ranges, these variations were not considered toxicologically significant or related to treatment.

2. Clinical chemistry

Lower than control glucose blood levels were noted in both males and females in all the dose groups, attaining statistical significance at above and including 3200 ppm in the males, with mean values up to -49% lower than control, and at all the dose levels tested in the females, with mean values of up to - 52%.

Cholesterol mean values increased in the 3200 ppm group and above to up to 65% in the males, attaining statistical significance at 8000 ppm (38%) and 20000 ppm (65%), and up to 49% in the females, attaining statistical significance in the High dose females only (49% higher than control).

Albumin and A/G ratio were slightly higher than control, attaining statistical significance at 8000 and/or 20000 ppm in the males and in both males and females, respectively.

These effects were considered to reflect a BYI 02960-difluoroethyl aminofuranone-related adverse effect at and above 3200 ppm, under the conditions of this study.

In addition, higher urea was noted in the 20000 ppm males (32%), with 20% and 15% higher values in the 20000, and 8000 ppm females, respectively, without attaining statistical significance. As the dose response was not clear, although a test item effect at 20000 ppm cannot be excluded, it cannot be ascertained in the conditions of this study. Similar changes that were considered equivocal in correlation with BYI 02960-difluoroethyl aminofuranone administration in diet mostly at 8000 and 20000 ppm were noted in the ALT, T-BIL and bile acids mean values, with statistically significant variations observed on occasion, although without a clear dose or gender response.

Other clinical chemistry parameters showed on occasion statistically significant variations, however, there was no dose or gender response or the values were within the physiological ranges. For this reason, these variations were not considered toxicologically significant or related to treatment.

Table 5.8-26: Summary of clinical chemistry changes

Dose levels of Test item (ppm)	0	1280	3200	8000	20000
Male					
Glucose mmol/L (%C)	4.24	3.92 (93%)	2.98** (70%)	2.15** (51%)	2.61** (62%)
Urea mmol/L (%C)	6.37	5.57 (87%)	5.91 (93%)	6.21 (98%)	8.41** (132%)
Cholesterol mmol/L (%C)	1.85	2.02 (110%)	2.42 (131%)	2.55* (138%)	3.06** (165%)
Albumin g/L (%C)	29.46	30.30 (103%)	30.38 (103%)	32.36** (110%)	36.56** (124%)
ALT U/L (%C)	49.40	52.20 (106%)	52.40 (106%)	56.80 (115%)	73.80* (149%)
Total bilirubin µmol/L (%C)	6.86	7.04 (103%)	6.66 (97%)	7.14 (104%)	8.62* (126%)
A/G	1.16	1.16 (100%)	1.20 (103%)	1.32** (114%)	1.52** (131%)
Bile acid µmol/L (%C)	12.68	11.76 (93%)	12.46 (98%)	12.48 (98%)	19.48** (154%)
Female					
Glucose mmol/L (%C)	5.54	4.35** (79%)	4.13** (75%)	2.66** (48%)	2.93** (53%)
Urea mmol/L (%C)	8.59	8.50 (99%)	8.61 (100%)	9.90 (115%)	10.35 (122%)
Cholesterol mmol/L (%C)	2.11	2.05 (97%)	2.71 (129%)	2.60 (123%)	3.15** (149%)
Albumin g/L (%C)	33.08	32.70 (99%)	35.00 (106%)	35.04 (106%)	36.25 (110%)
ALT U/L(%C)	50.00	56.40 (113%)	55.60 (111%)	51.60 (103%)	64.50 (29%)
Total bilirubin µmol/L (%C)	7.02	7.16 (102%)	6.94 (99%)	8.64* (123%)	9.73** (139%)
A/G	1.38	1.32 (96%)	1.42 (103%)	1.46 (106%)	1.58* (114%)
Bile acid µmol/L (%C)	11.54	13.12 (114%)	14.58 (126%)	16.50 (143%)	18.33 (159%)

* : $p < 0.05$ ** : $p < 0.01$

E. Sacrifice and pathology

1. Organ weights

At 8000 and 20000 ppm there were effects, many of which reflected the lower body weights in these animals, with lower absolute weights and unchanged or higher organ weights relative to body weight (generally reaching statistical significance) such as the heart, brain, kidney, liver, testes and epididymides. In males, the thymus, at 20000 ppm and prostate at 8000 and 20000 ppm were smaller than control even after adjustment for body weight.

At 3200 ppm there were no effects on organ weights in either sex that were consistent between absolute and adjusted organ weights, the statistical differences were not considered to reflect a clear effect of test item other than secondary to body weight differences.

There were no effects at 1280 ppm in either sex, a statistical difference in male kidney weights adjusted for bodyweight was within the normal range and not considered a clear test item related effect.

2. Gross and histopathology

Potentially test item-related macroscopic changes were observed at necropsy in the High dose group fed diet with 20000 ppm BYI 02960-difluoroethyl aminofuranone. These consisted of small prostate and seminal vesicles in 5/5 males and small spleen, in 2/5 males, were associated with the potential test item related changes in the terminal body weights and correlated with the organ weight changes, however, a definitive attribution cannot be made without histopathological evaluation.

Other observations were considered to be incidental or associated with morphological changes during oestrus cycle.

III. Conclusions

In conclusion, based on the effects noted in the current 14-day preliminary study and in correlation with the previous data available at the Sponsor, the dose levels selected were 200, 800 and 3000 ppm. These levels were considered to be suitable for an upcoming 28-day dietary study with BYI 02960-difluoroethyl aminofuranone.

Primary review: Australia
Secondary review: US EPA & PMRA

Report: Kubaszky R (2012) Final Report. BYI02960-difluoroethyl aminofuranone: A 28-Day Dietary Toxicity Study in Wistar Rats. CiToxLAB Hungary Ltd. (formerly LAB Research Ltd), H-8200 Veszprém, Szabadságpuszta, Hungary. Laboratory Report No**.:** 11/116-100P. 29 February, 2012. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: M-426136-01-2. MRID 48844110. Unpublished

Guidelines: OECD TG 407 (2008); EC Directive 96/54/EC B.7 (1996); EC No 440/2008 (2008); US EPA OPPTS 870.3050 (2000).

GLP: Yes. Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a 28-day dietary study (MRID 48844110), BYI02960-difluoroethyl aminofuranone was administered to groups of Wistar (Crl:WI) rats (10/sex/dose) in the diet in at concentrations of 0, 200, 800, or 3000 ppm (males: 0, 17, 67, and 244 mg/kg bw/d; females: 0, 19, 76, and 256 mg/kg/d) for 28 days.

Under the conditions of the study, there were no test substance related clinical signs, mortality, urinalysis parameters, organ weights, or evidence of neurotoxicity as determined by the Functional Observation Battery. There were some slight changes in hematological parameters (erythrocyte counts, hemoglobin, and prothrombin time) and clinical chemistry (plasma calcium), but the changes were small and were within expected ranges for the type and age of animals being used in the study. These changes were not considered as adverse. No adverse gross or histopathology changes were found.

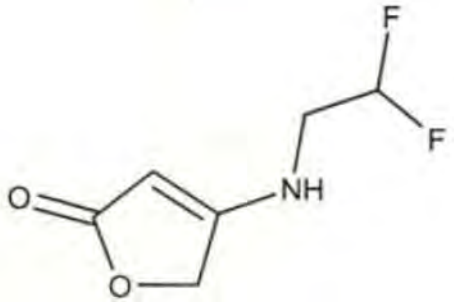
The NOAEL was 3000 ppm (244/256 mg/kg/day, M/F), the highest concentration tested. No LOAEL was established.

This study is fully reliable (acceptable/guideline) and meets the data requirements for a 28-day oral toxicity study (OPPTS 870.3050; OECD 407).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI02960-difluoroethyl aminofuranone



Formula:	4-[(2,2-difluoroethyl)amino]furan-2(5H)-one
Lot/Batch #:	NLL 8671-12-1
Purity:	98.9% (25 July 2011, 1H-NMR)
Description:	White powder
CAS #	Not reported
Stability of test compound:	Stated to be six months if stored at 20±10ºC. Homogeneity and concentration checks of BYI02960-difluoroethyl aminofuranone in the prepared formulations performed by HPLC-UV were in the range of 91 and 107% of nominal concentrations.

2. Vehicle and/or positive control: Untreated diet

3. Test animals

Species:	Rats
Strain:	Wistar (Crl:WI)
Age at dosing:	At least seven weeks old
Weight at dosing:	243 to 287 g (males) and 173 to 213 g (females)
Source:	Charles River Laboratories, Research Models and Services, Germany GmbH, Sandhofer Weg 7, D-97633
Acclimation period:	14 days
Diet:	ssniff® SM R/M-Z+H “Autoclavable Complete Feed for Rats and Mice – Breeding and Maintenance” (ssniff Specialdiäten GmbH Ferdinand-Gabriel-Weg 16 Soest D-59494 Germany), *ad libitum*, except for overnight fasts prior to blood sampling and scheduled necropsy.
Water:	Tap water, *ad libitum*, except for overnight prior to the day of sacrifice.
Housing:	Within dosing groups at 5/sex/cage, with deep wood sawdust bedding, to allow digging and other activities.

4. Environmental conditions

Temperature:	19.7 to 23.6°C
Humidity:	30 to 48%
Air changes:	15 to 20 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. In-life initiated/completed

   3 November to 2 December 2011

2. Animal assignment and treatment

   Details of animals and dosing levels are shown in Table KIIA 5.8.14/01. Three groups of 10 Wistar rats/sex/dose received concentrations of 0, 200, 800, or 3000 ppm BYI02960-difluoroethyl aminofuranone in feed daily for 28 days. The dosing regimen was selected based on results from a previous 14-day palatability study conducted in rats with this test substance (MRID 48844109), where treatment-related effects observed included decreased serum glucose in females at ≥ 1280 ppm and males at ≥ 3200 ppm, decreased bodyweight gain in both sexes at ≥ 3200 ppm, and decreased food consumption at ≥ 8000 ppm.

   Animals were assigned to dose groups by computerised, stratified randomisation so that there was less than a 20% difference among group bodyweights at onset of dosing. A control group of male animals received diet free of the test substance only.

   Table KIIA 5.8.14/01: Study design

nominal conc. in diet (ppm)	achieved mean daily intake (mg/kg bw/d)				animal ID No's [N=10]	
	M		F			
	Mean	Weeks 1 to 4 range	Mean	Weeks 1 to 4 range	M	F
0	-	-	-	-	1001 to 1010	1501 to 1510
200	17±1	14 to 19	19±1	17 to 20	2001 to 2010	2501 to 2510
800	67±3	57 to 78	76±8	67 to 83	3001 to 3010	3501 to 3510
3000	244±13	214 to 276	273±8	256 to 292	4001 to 4010	4501 to 4510

Note: Test substance was BYI02960-difluoroethyl aminofuranone. Achieved dosing does not account for test substance purity or degradation. Nominal dosing was intended to be approximately 0, 10, 40, and 150 mg/kg bw/d. Data excerpted from pages 11 of the study report.

3. Diet preparation and analysis

   BYI02960-difluoroethyl aminofuranone was added directly to the diet and mixed by mechanical mixers of a precision stated to be at least 1:100000, for up to approximately 14 minutes under temperature and humidity controlled conditions. Following incorporation into diet, the feed with admixed test substance was pressed into pellets by simple compression, and stored at 15 to 21°C and low humidity until use. Two batches were made for each dosing concentration, the first batch were supplied to animals on weeks 1 to 3, and the second batch on week 4. Homogeneity and concentration checks of BYI02960-difluoroethyl aminofuranone in the prepared formulations performed by HPLC-UV were in the range of 91 and 107% of nominal concentrations, with measured concentrations in week 1-3 batch of 98% (200 ppm), 91% (800 ppm), and 93% (3000 ppm), while the week 4 batch was 101% (200 ppm) and 107% (800 ppm and 3000 ppm) of nominal concentrations. The prepared formulations were therefore considered acceptable for use.

   In a separate study (Study Code 11/116-316AN), the stability of BYI02960-difluoroethyl aminofuranone in the diet in storage room conditions (15 to 21°C) was evaluated at 500–20000 ppm and found to be stable for at least seven weeks, while in animal housing room

conditions (22±3ºC), test material in prepared diet at 500–20000 ppm was stable for at least 14 days. Based on these results, a single batch of diet was prepared for each concentration at the study outset for use during the study.

4. Statistics

Mean and standard deviations values, the frequency of clinical observations and pathology findings were calculated as applicable. Statistical evaluation of numerical data was performed with PSS PC+4.0 software (SPSS Hungary Kft, Budapest). Heterogeneity of variance between groups was checked by Bartlett's homogeneity of variance test. Where no significant heterogeneity was detected, a one-way analysis of variance was carried out. If the obtained result was positive, Duncan's Multiple Range test was used to assess the significance of inter-group differences. Where significant heterogeneity was found, the normal distribution of data was evaluated by Kolmogorov-Smirnov test. In case of abnormal distribution, the nonparametric method of Kruskal-Wallis One-Way analysis of variance was applied. If the result was positive, the inter-group comparisons were performed using Mann- Whitney U-test.

C. METHODS

1. Observations, including Functional Observational Battery testing

Animals were observed at least twice daily for mortality and morbidity. Detailed clinical observations were undertaken at 7 days prior to dosing and on a weekly basis thereafter. Cages and cage-trays were inspected daily. The oestrus cycle of all females was evaluated by examination of vaginal smears prior to scheduled necropsy.

Detailed clinical observations included examination of skin, fur, eyes, eyeballs and mucous membranes, autonomic activity (lachrymation, piloerection, pupil size, respiratory pattern, occurrence of secretions and excretions), circulatory and central nervous system (tremor, convulsion, muscular contractions), somatomotor activity and behaviour pattern (changes in exploratory behaviour, ordinary behaviour including changes in grooming, headshaking, gyration, abnormal behaviour such as self-mutilation, backward motion, abnormal vocalization, aggression), motor coordination, ambulatory abnormalities, changes in body position and posture (ex. hunchback posture), gait, or response to handling and to environmental stimulation. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Additionally, animals were subjected to Functional Observational Battery examination on study day 26. This consisted of qualitative assessment of the grip strength, and measurements of landing foot splay and fore/hind grip strength, described as follows:

- Landing foot splay was measured by painting the hind/fore paws and dropping animals from a horizontal position onto a covering sheet and measuring the distance between the two resulting ink spots.

- Fore/hind grip strength measurements were conducted using a grip strength meter (Model GS3, Bioseb, Chaville, France) where rats were allowed to grip the support bar with their forelimbs and pull back until releasing the bar at least three times per animal and the procedure was then repeated with the hind limbs.

Additionally, sensory reactivity to different type of stimuli (*e.g.*, auditory, visual and

proprioceptive) were measured and the general behaviour of animals examined. A modified Irwin test (Irwin, 1968. Psychopharmacologia Berl. 13:222-257) was performed and provided sensitive detection of neurotoxicity symptoms effects including body position, locomotor activity, respiration rate, respiration type, piloerection, head searching compulsive biting or licking, circling, upright walking, retropulsion, jumping, exophthalmos, twitches, clonic convulsions, tonic convulsions, tremor, startle, transfer arousal, spatial locomotion, gait, posture, limb position, finger approach, finger withdrawal, touch escape response, diarrhoea, diuresis, visual placing, body tone, corneal reflex, pinna, toe pinch, grasping reflex, positional struggle, skin, mucous membrane colour, salivation, palpebral closure, lachrymation, limb tone, abdominal tone, tail pinch, righting reflex, and/or vocalisation were evaluated.

2. Body weights

All animals were weighed at study initiation and at necropsy, and on a weekly basis over the study period.

3. Food consumption, food efficiency, and daily intake

Food consumption was recorded on a weekly basis. The mean achieved test substance dosing was defined using the following formula:

$$\text{intake (mg/kg bw/d)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption per week (g/day)}}{\text{Group mean body weight (g) per week}}$$

Food conversion efficiency (g/g) was calculated using the formula: weekly body weight gain (g)/weekly food consumption (g).

4. Clinical pathology (clinical chemistry and haemotology)

On study day 28, animals were removed from metabolism cages and sacrificed by via heart puncture exsanguination under pentobarbital anaesthesia. Exuded blood was collected and stored in stored in tubes containing EDTA (1.2 mL) for haematology, plain tubes (2.6 mL) for plasma chemistry, or sodium citrate (1.6 mg/mL) for coagulation parameter analysis.

The following haematology parameters were measured using an Advia 120, except for prothrombin and activated partial thromboplastin times, which were assessed using an AMAX Destiny Plus Coagulometer. Clinical chemistry parameters were examined by a VITROS 250 (also labelled as VITROS 950 in error in the report). Blood smear examination was undertaken only if abnormal haemotology results were reported with Advia 120 analysis.

The following parameters were examined:

Hematology and coagulation

Red blood cell count	Hemoglobin
Red blood cell volume	Reticulocyte count
Hematocrit	Platelet count
Mean platelet volume	Mean corpuscular (cell) volume
Mean corpuscular (cell) hemoglobin	
Mean corpuscular (cell) hemoglobin concentration	
White blood cell count (total and differential)	

Prothrombin time
Activated partial thromboplastin time
Microscopic blood smear examination

Clinical chemistry parameters

aspartate aminotransferase	total protein
alanine aminotransferase	albumin
alkaline phosphatase	total bilirubin
gamma glutamyltransferase	bile acids
urea	A/G ratio
total cholesterol	triglycerides
glucose	creatinine
phosphorous	sodium
potassium	calcium
chloride	

5. Urinalyses

On study day 27, animals were placed in individual metabolism cages to collect overnight urine samples (16 hours), and food and water was withheld. Measurements were made of urinary volume, specific gravity (METTLER PG 203-S), sediment (Leitz Microscope), and (all by Uryxxon 300) pH, glucose, nitrogen, bilirubin, ketone, protein and urobilinogen, and presence of red or white blood cells.

6. Sacrifice and pathology

At termination, animals were subjected to gross necropsy. Organs that were weighed are listed in KIIA 5.8.14/02. Organ weight/final body weight and organ weight/brain weight ratios were calculated. Initially, tissues from all animals in the highest dose and control groups were histopathologically examined, and only if a given tissue was abnormal at the highest dose was examination continued for all animals from all dosing groups.

Table KIIA 5.8.14/02. Organs/tissues collected for pathological examination

Organ	Weighed	Histopathological evaluation conducted[a]
Adrenal gland	X	X
Aorta	-	X
Brain (cerebral cortex, midbrain, cerebellum and medulla)	X	X
Epididymides	X	X
Eyes with optic nerves	-	X
External lachrymal glands	-	X
Femur with marrow	-	X
Gross observations	-	X
Harderian glands	-	X
Heart	X	X
Kidneys	X	X
Large intestines (cecum, colon and rectum)	-	X
Liver	X	X
Lungs (with bronchi)	-	X
Lymph nodes (mandibular and mesenteric)	-	X
Mammary glands	-	X
Oesophagus	-	X
Ovaries with oviduct	X	X
Pancreas	-	X
Pituitary	-	X
Prostate	X	X
Salivary glands (including mandibular, sublingual and parotid glands)	-	X
Sciatic nerve	-	X
Seminal vesicles with coagulating glands	X	X
Skeletal muscle (quadriceps)	-	X
Skin & subcutis (inguinal)	-	X
Small intestines (duodenum, ileum and jejunum with Peyer's patches.)	-	X
Spinal cord (cervical, lumbar, and thoracic)	-	X
Spleen	X	X
Sternum with marrow	-	X
Stomach	-	X
Testes	X	X
Thymus	X	X
Thyroid gland (with parathyroid glands)	X	X
Tounge	-	X
Trachea	-	X
Urinary bladder	-	X
Uterus including horns, body and cervix	X	X
Vagina	-	X

[a] Tissues from all animals in the highest dose and control groups were histopathologically examined initially, and if a given tissue was abnormal, examination continued for all animals from all dosing groups.

II. RESULTS AND DISCUSSION

A. GENERAL OBSERVATIONS

1. Observations including Functional Observational Battery testing

   There were no test substance related clinical signs and no evidence of neurotoxicity as determined by the Functional Observation Battery.

   Minor variations were observed throughout the groups, on occasion attaining statistical significance, for example slightly lower landing foot splay mean values for forelimbs in males at 800 and 3000 ppm, or slightly higher grip strength for hindlimbs in females at 200 and 3000 ppm females. They were regarded as incidental, as no dose or gender consistent response was observed.

2. Mortality

   There were no unscheduled mortalities.

B. BODY WEIGHT AND BODY WEIGHT GAIN

All groups experienced mean group bodyweight gains over each of weeks 1 to 4, however, all test substance group females and in males at 3000 ppm, slight non- statistically significant (*i.e.*, $p \geq 0.05$) decreases in the rate of bodyweight gain relative to controls were seen in at least one dosing group in all but study week 2 for females (Table KIIA 5.8.14/03). At 200 and 800 ppm, overall group mean study day 0 to 27 bodyweight gains were 4 to 6% higher than those experienced by controls, while gains in males at 3000 ppm were 12% lower relative to those in controls (statistically insignificant, *i.e.*, $p \geq 0.05$).

In females, all test substance groups experienced relatively smaller bodyweight gains but this effect displayed poor dose dependency, with group mean bodyweight gains over study days 0 to 27 being 19 (200 ppm), 5 (800 ppm), and 13% (3000 ppm) lower than that in controls.

The relatively lower bodyweight gains over day 0 to 27 at 3000 ppm in both sexes included 18/32% lower gains on day 1 to 7 versus those in controls.

The bodyweight data were regarded by the study authors as possibly being test substance related, but no adverse due to their slight extent (5% lower final bodyweight in both sexes at 3000 ppm relative to controls) and lack of statistical significance (*i.e.,* p was ≥ 0.05). However, the study authors regarded the findings as being consistent with the 15-20% lower bodyweight gains relative to controls observed over 14 days in rats receiving dietary concentrations of 8000 ppm BYI02960-difluoroethyl aminofuranone (CiToxLAB study code 11/116-100PE). The evaluator consideres that the the bodyweight changes were not test substance related, based on the absence of dose dependency.

Table KIIA 5.8.14/03 Mean bodyweight gains

N= 10	bodyweight gain (g) or % change from control+SD (as % of control mean)							
	Males				Females			
Study day interval	Dose (ppm)							
	0	200	800	3000	0	200	800	3000
Day 0 starting bodyweight (g)	*263.4±10.1*	*268.9±12.0*	*264.2±8.0*	*260.2±10.3*	*197.2±9.4*	*199.4±8.4*	*193±11.3*	*192.5±7.2*
1 to 7 gain	51.9±6.2	50.2±8.2 (-3%)	53.4±7.8 (+3%)	42.7±8.7* (-18%)	28.6±7.1	18±7.8** (-37%)	21.1±7* (-26%)	19.5±4.6** (-32%)
8 to 14 gain	37.7±10.4	50.8±14.4* (+35%)	47.6±8.9* (+26%)	34.1±5.8 (-10%)	11.1±7.9	14.8±8.3 (+33%)	16.4±5.4 (+48%)	12.2±6.5 (+10%)
15 to 21 gain	38.4±7.8	39.9±9.6 (+4%)	37.4±7.3 (-3%)	33.7±6.6 (-12%)	16.8±5.8	10.3±4.8* (-39%)	19.1±8.9 (+14%)	12.7±3.3 (-24%)
22 to 27 gain	20.9±9.2	27.8±7.7 (+33%)	24.3±8.5 (+16%)	20.1±8.6 (-4%)	8.3±7.2	9.3±6 (+12%)	4.8±7.5 (-42%)	11.8±6.9 (+42%)
0 to 27 gain	148.9±19.6	168.7±32.7 (+13%)	162.7±25.2 (+9%)	130.6±21.9 (-12%)	64.8±12.1	52.4±9.1 (-19%)	61.4±15.3 (-5%)	56.2±15 (-13%)
Day 27 bodyweight (g)	412.3±25.5	437.6±40.7 (+6%)	426.9±29.7 (+4%)	390.8±27.7 (-5%)	262.0±18.4	251.8±13.0 (-4%)	254.5±25.1 (-3%)	248.7±15.1 (-5%)

'*' denotes values that are statistically significant at the p≤0.05 or ** p≤0.01 level. Underlined denotes instances of lower bodyweight gain relative to control. Data excerpted from pages 68 to 71 of the study report

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

Relative to controls over study days 0 to 27, average group mean food consumption was decreased in males at 3000 ppm only, and decreased in females in a poorly dose dependent manner across all treatment groups, with the majority of the decreases occurring in study weeks 1 and 2 (7% to 15% decreases across all test substance group females). The study authors did not regard any of the food consumption findings to be toxicologically significant.

Table KIIA 5.8.14/04: Mean food consumption

N= 2 only	consumption (g/animal/d+SD in g) or % change from control)							
	Males				Females			
Study day interval	Dose (ppm)							
	0	200	800	3000	0	200	800	3000
-7 to 0	26.3±1.4	26.8±1	26.5±0.2	25.5±0.4 **(-3%)**	21±**1.3**	20.7±0.8	21±0.7	20.8±0.5 **(-1%)**
1 to 7	28.1±0.4	28.5±0	28.4±0.1	25.9±0.1 **(-8%)**	22.7±**0.4**	21.2±0.1 **(-7%)**	21.1±0.3 **(-7%)**	19.7±0.8 **(-13%)**
8 to 14	28.7±1.9	30±1.1	29.8±0.5	26.9±0.2 **(-6%)**	23.2±**1**	20.7±0.8 **(-11%)**	21.6±2.2 **(-7%)**	19.7±1.1 **(-15%)**
15 to 21	28±1.8	29.1±1.7	28.7±0.2	26.3±0.3 **(-6%)**	22.3±**0.4**	20.9±0.3 **(-6%)**	21.7±1.4 **(-3%)**	20.2±1.1 **(-9%)**
22 to 27	28.4±1.8	29.6±1.5	29.6±0.2	27.2±0.3 **(-4%)**	21.9±**0.5**	21±0.5 **(-4%)**	21.1±0.5 **(-4%)**	20.7±1.4 **(-5%)**
0 to 27	27.9+	28.8+2.7	28.6+0.6	26.4+0.6 **(-6%)**	22.22+1.8	20.9+1.3 **(-6%)**	21.3+2.8 **(-4%)**	20.2+2.3 **(-9%)**

Statistical analyses were not undertaken. Underlined denotes instances of per cent decrease food consumption relative to control. Data excerpted from pages 74 and 75 of the study report, and per cent changes (rounded to integers) were calculated manually by the evaluator using Microsoft Excel.

Relative to controls, overall weeks 1 to 4 food use efficiency was decreased by 6% in males at 3000 ppm. However, these animals experienced a 12% decrease in food use efficiency in the acclimation phase week prior to dosing, versus only a 10% decrease in week 1 relative to controls (Table KIIA 5.8.14/05).

Relative to controls, overall food use efficiency was decreased by 32% (200 ppm) to 21% (3000 ppm) in females in study week 1, but recovered in weeks 2 and 4 to reach overall food use efficiencies of -12% (200 ppm), no change (800 ppm), and -2% (3000 ppm) relative to controls. The study authors regarded these findings as being unrelated to the test substance. This conclusion was considered to be supported when considered in conjunction with the overall lack of treatment-related change in bodyweight gains across test substance animals.

Table KIIA 5.8.14/05: Food use efficiency ([weekly body weight gain (g)/weekly food consumption (g))

N= 2 only Study day interval	Efficiency (bw gain/food consumption) and % change from control (bolded)							
	Males				Females			
	Dose (ppm)							
	0	200	800	3000	0	200	800	3000
-7 to 0	0.28	0.3	0.28	0.24	0.18	0.2	0.17	0.16
% Δ cntrl		**+8.08**	**+0.41**	**-12.15**		**11.48**	**-2.66**	**-12.32**
1 to 7	0.26	0.25	0.27	0.24	0.18	0.12	0.14	0.14
% Δ cntrl		**-4.6**	**1.8**	**-10**		**-32.61**	**-20.63**	**-21.44**
8 to 14	0.19	0.24	0.23	0.18	0.07	0.1	0.11	0.09
% Δ cntrl		**+28**	**21.6**	**-3.5**		**+49.44**	**+58.69**	**29.44**
15 to 21	0.2	0.2	0.19	0.18	0.11	0.07	0.13	0.09
% Δ cntrl		**-0.02**	**-4.98**	**-6.57**		**-34.58**	**+16.83**	**-16.55**
22 to 27	0.12	0.16	0.14	0.12	0.06	0.07	0.04	0.1
% Δ cntrl		**+27**	**11.55**	**0.42**		**16.85**	**-39.98**	**+50.41**
0 to 27	0.19	0.21	0.21	0.18	0.11	0.09	0.11	0.1
% Δ cntrl		**+9.46**	**6.68**	**-6.28**		**-12.96**	**-0.09**	**-2.47**

Standard deviations not provided by the study author and were not calculated by the evaluator. Data excerpted from page 76 of the study report. Per cent change values could not be replicated by evaluator, with differences likely to be due to the study author's calculations proceeding prior to rounding to two decimal places in the study report.

D. CLINICAL PATHOLOGY

1. Clinical pathology – haematology

At 200 ppm in males, statistically significant increases of 70% ($p \leq 0.05$) in white blood cells, 5% ($p \leq 0.01$) in erythrocytes, 4% ($p \leq 0.05$) in haemoglobin, and 54% ($p \leq 0.05$) in monocytes were seen relative to controls. Of these changes, only haemoglobin demonstrated any dose concordance, with 6% ($p \leq 0.01$) and 8% ($p \leq 0.01$) increases relative to controls at 800 and 3000 ppm, respectively. At 800 ppm in males, a 6% ($p \leq 0.01$) increase was seen in prothrombin time relative to controls, and the effect increased in severity to a 10% increase ($p \leq 0.01$) at 3000 ppm. In females, statistically significant changes in clinical chemistry parameters were confined to increases of 71% ($p \leq 0.01$) and 85% ($p \leq 0.01$) in white blood cell counts at 800 ppm and 3000 ppm, respectively, and a 2% ($p \leq 0.01$) increase in mean corpuscular haemoglobin concentration at 3000 ppm (Table KIIA 5.8.14/06).

The study authors did not regard any clinical chemistry findings to be test substance related, on the basis of being within expected physiological ranges, the results being largely being confined to males, and poor dose dependencies. However, historical control data were not provided in the report.

Based on the biological consistency of the findings, with increased red blood cells, haematocrit, haemoglobin, and mean corpuscular (at 3000 ppm only) hemoglobin concentration, all of statistical significance ($p \leq 0.05$), the evaluator considers that test

substance related increases in erythrocyte production and haemoglobin synthesis occurred in males at 800 ppm and above. However as increases rather than decreases, these findings in isolation and at the proportions described (less than 10% increases) are not considered to be toxicologically adverse in rats, nor are they considered to be potentially adverse in humans.

Table KIIA 5.8.14/06: Haemotology

	Dose (ppm)							
	M				F			
N=10	**0**	**200**	**800**	**3000**	**0**	**200**	**800**	**3000**
WBC (K/uL)	4.494±2.153	7.627±2.286* **(+70%)**	8.029±2.171** **(+79%)**	6.559±3.175 **(+46%)**	2.96±1.391	3.797±1.528 **(+28%)**	5.065±1.429** **(+71%)**	5.482±1.727** **(+85%)**
RBC (M/µL)	8.08±0.385	8.513±0.178** **(+5%)**	8.477±0.267** **(+5%)**	8.648±0.348** **(+7%)**	8.231±0.33	8.131±0.29	8.266±0.265	8.323±0.345
HGB (g/dL)	14.53±0.5	15.17±0.38* **(+4%)**	15.47±0.66** **(+6%)**	15.68±0.55** **(+8%)**	15.13±0.58	15.08±0.54	15.37±0.67	15.14±0.45
Hct (%)	44.43±1.84	45.77±1.46	46.5±1.75* **(+5%)**	46.51±1.77* **(+5%)**	45±1.72	44.55±1.8	45.89±1.55	44.12±1.15
MCHC (g/dL)	32.7±0.51	33.15±0.8	33.25±0.83	33.7±0.37** **(+3%)**	33.63±0.45	33.85±0.61	33.49±0.54	34.3±0.49** **(+2%)**
Mono (%)	2.11±0.61	3.25±0.866 **(+54%)**	3.25±1.536* **(+54%)**	3.32±1.053 **(+57%)**	2.83±0.625	3.08±1.491 **(+9%)**	2.87±0.97 **(+1%)**	3.39±1.004 **(+20%)**
PTT(s)	20.87±0.67	21.19±0.71 **+2**	22.18±0.56** **(+6%)**	23.06±0.63** **(+10%)**	20.86±1.21	20.92±1	20.96±1.13	21.41±0.71

'*' denotes values that are statistically significant at the $p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from pages 77 to 82 of the study report, except for per cent changes (rounded to integers) which were calculated by the evaluator using Microsoft Excel.

2. Clinical pathology – clinical chemistry

Statistically significant ($p \leq 0.05$) changes in clinical chemistry parameters were observed at ≥ 800 ppm in both sexes. In females at 800 ppm, calcium was increased by 3% relative to controls ($p \leq 0.05$), increasing to 9% ($p < 0.01$) above controls at 3000 ppm and also being 13% ($p < 0.05$) above controls in males at this dose. All other findings in females at 800 ppm displayed poor dose dependency, with the severity of effects all being lower at 3000 ppm, with the exception of total protein which remained at a 7% decrease ($p \leq 0.01$) and also being decreased by 3% ($p < 0.05$) in males at 3000 ppm (Table KIIA 5.8.14/07).

There were no other statistically significant findings with at least slight dose dependency and/or consistent direction of change between sexes.

Based on the absence of dose dependency, correlation between sexes, and inter-individual variability, the study authors considered the clinical chemistry findings to be unrelated to the test substance. The evaluator agrees with the study author.

Table KIIA 5.8.14/07: Clinical chemistry

	Dose (ppm)							
	M				F			
N=10	0	200	800	3000	0	200	800	3000
Creat. (µmol/L)	56.96±5.86	51.75±6.69	60.02±9.78	61.54±5.85	54.19±5.35	58.18±5.42	58.16±7.82	47.96±3.48* **(-11%)**
Phos. (mmol/L)	2.671±0.335	2.693±0.15	2.925±0.205* **(+10%)**	2.937±0.185* **(+10%)**	2.466±0.238	2.575±0.244	2.433±0.241	2.86±0.164** **(+16%)**
Na+ (mmol/L)	144.51±1.1	144.65±1.29	145.46±0.97	144.84±0.78	155.81±2	155.84±1.31	148.5±1.66** **(-5%)**	152.1±2.11** **(-2%)**
K+ (mmol/L)	4.718±0.454	5.173±0.548	5.008±0.534	5.313±0.398* **(+13%)**	4.93±0.481	5.26±0.353	4.99±0.26	5.71±0.714* **(+16%)**
Ca++ (mmol/L)	2.524±0.045	2.569±0.06	2.567±0.065	2.648±0.068** **(+5%)**	2.557±0.104	2.543±0.063	2.627±0.05* **(+3%)**	2.79±0.056** **(+9%)**
Cl- (mmol/L)	100.81±2.12	102.52±2.03	101.04±1.71	100.73±1.23	110.65±1.5	110.82±1.81	104.84±1.81** **(-5%)**	107.2±2.31** **(-3%)**
Tot.Prot (g/L)	56.36±1.18	56.24±1.99	55.1±1.13	54.41±1.88* **(-3%)**	63.33±3.29	60.77±3.53	58.62±2.05** **(-7%)**	58.75±3.34** **(-7%)**
Alb. (g/L)	31.32±1.01	31.13±0.97	30.96±0.82	29.94±1.31** **(-4%)**	35.54±4	34.05±2.51	31.52±2.11* **(-11%)**	34.62±4.25
AST/GOT (U/L)	198.4±75.8	157±49.5	215.3±75.5	138.1±32.2* **(-30%)**	200.5±64.7	240.1±102	191.1±69.8	272.7±92.1
Bile Acid (µmol/L)	14.41±2.461	11.79±3.616	17.68±3.681* **(+23%)**	11.75±3.246	12.59±4.114	16.53±5.135	16.85±5.323	20.9±3.952** **(+66%)**

*: denotes values that are statistically significant at the $p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from pages 84 to 87 of the study report, except for per cent changes (rounded to integers) which were calculated by the evaluator using Microsoft Excel.

4. Urinalysis

Urinalysis parameters were unaffected by the test substance, with no findings being statistically significant ($p \leq 0.05$) and/or demonstrating dose dependency.

F. SACRIFICE AND PATHOLOGY

1. Organ weight

There were no test substance related changes in absolute or relative (to brain or bodyweight) weights of organs.

Incidental changes including bilateral enlargement of the adrenals, dilatation of the renal pelvis and enlargement and/or discoloration of the prostate were noted with low incidence and/or throughout all the dose groups, unrelated to treatment.

No statistically significant differences were recorded in absolute mean organ weights, and no changes in organ weights relative to brain weight reached statistical significance ($p \leq 0.05$) at 3000 ppm, though minor incidental increases in relative liver weights were observed in

800 ppm females and in relative testes weights in 800 ppm males. Statistically significant variations in organ weights relative to bodyweights were observed with poor dose dependency, and included slightly (~5% in both sexes) increased relative kidney weights in both sexes at 3000 ppm, and increased relative testes (7%) and seminal vesicles (12%) weights in males at 3000 ppm.

The study authors regarded the organ weight findings as being unrelated to the test substance as the values were within the normal ranges and/or of low magnitude, showed no consistent response between genders, and/or were not correlated with pathological findings. Based on the absence of changes in absolute weights or relative to brain weight changes at 3000 ppm (the highest dose tested in the study), the evaluator agreed with the study author that there were no test substance related changes on organ weights.

2. Gross pathology and histopathology

Incidental changes in gross necropsy findings including bilateral enlargement of the adrenals, dilatation of the renal pelvis and enlargement and/or discoloration of the prostate were noted with low incidence and/or without dose dependence, and overall were of an incidence regarded by the study authors as being within expected natural variations.

Histopathology findings were confined to minimal (N=1) or mild (N=1) focal hepatocellular necrosis in 2/10 males at 3000 ppm, but this effect was not present in females which received slightly higher dose (256 mg/kg/d) than males (244 mg/kg/d). No adverse clinical chemistry changes were seen in any test groups. Therefore, this finding was not considered as adverse.

III. CONCLUSION

Under the conditions of this study, BYI02960-difluoroethyl aminofuranone did not produce test substance-related related clinical signs, mortality, urinalysis parameters, or organ weights, and no evidence of neurotoxicity as determined by the Functional Observation Battery.

The NOAEL was 3000 ppm (244/256 mg/kg bw/d for M/F), the highest dose tested. No LOAEL was established.

This study is reliable (acceptable/guideline). The study yields useful information concerning the toxicity of this chemical and a NOAEL can be established. Therefore it meets the guideline requirement for a short term toxicity study (OPPTS 870.3050; OECD 407).

Primary review: Australia
Secondary review: US EPA & PMRA

Report: Odin-Feurtet M (2009) BYI 0260. 90-day toxicity study in the rat by dietary administration. Bayer CropScience, 355, rue Dostoievski, 06903 Sophia Antipolis Cedex, France. Laboratory Report No.: SA 07294. 10 February 2009. MRID 48844111. PMRA 2239420. Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N°TXRVP008. Bayer CropScience File No. M-329048-03-2. Report Amendment Nº 1 date: 27 July 2011. Report Amendment Nº 2 date: 21 March 2012.

Guidelines: OPPTS 870.3100 (1998)
OECD 408 (1998)
Commission Directive 2001/59/EC Part B.26 (2001)
MAFF 12 Nousan 8147 (2000)

Deviations: A female (Animal No. ST4F0499; 2500 ppm) was not subjected to an ophthalmological examination at Study Day 91 in error. This deviation was not considered to affect the reliability of study results.

GLP: Yes. Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a subchronic dietary study (MRID 48844111), BYI 02960 (99.5%; NLL 7780-44-6) was administered to six to seven week-old Wistar Rj:WI (IOPS HAN) rats in groups of 10 rats/sex/dose at 0, 100, 500, or 2500 ppm daily for 95 to 97 days (0/0, 6.0/7.6, 30.2/38.3, and 156/186 mg/kg bw/d for M/F, respectively). Two additional groups (10 rats/sex/dose) received 0 or 2500 ppm for the same treatment period, then received diet free of the test substance for an additional 30 or 31 day recovery period.

There were no test substance related effects on clinical signs or FOB parameters, unscheduled mortality, ophthalmology, or urinalysis parameters.

The study also examined test substance concentrations in plasma during the last week of the study; the results indicated a linear increasing trend between plasma concentration vs dose level administered between 100 and 500 ppm, but a sub-linear increasing trend between 100 and 2500 ppm plasma concentrations of test substance. A slight sex-specific difference in test substance plasma concentrations was noted, with higher levels observed in females (1.3–1.7× higher than males).

At 2500 ppm, decreases in mean absolute body weight and body weight gains were observed in both sexes during the treatment period. During day 8 measuring interval, the absolute body weight decrement in females reached 10%, and at day 14 the decrease was 8%. Thereafter, the decrease in body weight was below 8% and persisted until the end of the study. The slight body changes were not considered adverse in males; for females, the decrease (↓10%) reached the magnitude of being adverse at the beginning of the study. The adverse body weight effect in females might reflect higher concentrations of the test compound in females relative to males. The food consumption

was consistently decreased in both males and females (↓9% to ↓29%). The increased incidence of gross and histopathological changes in the thyroid (dark color thyroid and thyroid follicular cell hypertrophy) combined with increased absolute and relative thyroid weights are considered to be adverse.

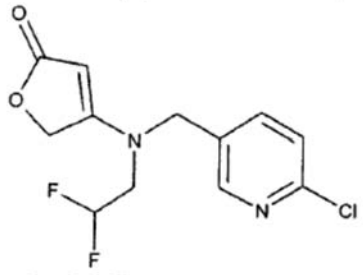
No adverse effects were found at 100 and 500 ppm.

Therefore, the NOAEL in both sexes was 500 ppm (38 mg/kg bw/day); LOAELwas 2500 ppm (156 mg/kg bw/day) based on increased incidence of thyroid follicular cell hypertrophy with associated increases of absolute and relative thyroid weights, decreased body weights in females, and decreased food consumption in both sexes.

This study is classified as fully reliable (acceptable/guideline) and satisfies guideline requirements for a subchronic oral toxicity in rats [OPPTS 870.3100; OECD 408 (1998)].

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	BYI 02960 4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
		
	Lot/Batch #:	NLL 7780-44-6
	Purity:	99.5%
	Description:	Pink powder
	CAS #	951659-40-8
	Stability of test compound:	Stated to be at least six months if stored as recommended at 25±5ºC (date of analysis 16 Jan 2008). Homogeneity concentration analyses in prepared feed found achieved concentrations of 93–96%. Stability analysis of the test substance reported variable results, with a number of feed samples stored at room temperature with 20 and 100 ppm test material below target values of 85% test material. However, the key 2500 ppm diet (the top dose in this study) was considered sufficiently stable (94% at day 0, 86% at day 102) for the purposes of the study. Prior freezing at -20°C for up to 92 days before being left at room temperature for 10 days did not result in test substance degradation rates greater than storage for 10 days at room temperature alone.
2.	Vehicle and/or positive control:	Untreated diet. No positive control in this study.
3.	Test animals	
	Species:	Rat
	Strain:	Wistar Rj:WI (IOPS HAN)
	Age at dosing:	6 to 7 weeks old
	Weight at dosing:	206 to 235 g for males; 158 to 187 g for females
	Source:	R. Janvier, Le Genest St Isle, France.
	Acclimation period:	9 days
	Diet:	A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*.
	Water:	Filtered and softened tap water, *ad libitum*
	Housing:	By sex in groups of 5 unless reduced by mortality or isolation. Housed in suspended, stainless steel and wire mesh cages.
4.	Environmental conditions	
	Temperature:	20 to 24°C

Humidity:	40 to 70%
Air changes:	10 to 15/hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion

   11 February 2008 to 10 December 2008 (main study).

   Blood samples analysed for concentration of BYI 02960 in plasma were undertaken between 28 September – 20 October 2011.

2. Animal assignment and treatment

   The dosing regimen of 0, 100, 500, or 25000 ppm was based on results from the rat 28-day oral gavage study (Capt, 2009a, Study No. SA 06075); the no-observed-adverse-effect-level (NOAEL) in both sexes was determined to be 75 mg/kg bw/day by the study author (and the evaluator).

   Four groups of 10 or 20 Wistar Rj:WI (IOPS HAN) rats/sex/dose were administered BYI 02960 at 0, 100, 500, or 2500 ppm in feed daily for 95 to 97 days, and mean doses were determined to be 0, 6.0, 30.2, and 156 mg/kg bw/day in males, and 0, 7.6, 38.3, and 186 mg/kg bw/day in females, respectively. The 0 and 2500 ppm dose groups contained 20 rats/sex, as 10 rats/sex represented a recovery sub-group that were sacrificed following a 30 or 31 day recovery period after termination of treatment (Table KIIA 5.3.2/01-1).

   Animals were assigned to dose groups by computerised, stratified randomisation, such that bodyweights were within 20% of the mean bodyweight on the day of randomisation. Animal housing and husbandry were in accordance with the provisions of the *Guide to the Care and Use of Laboratory Animals* (USPHS-NIH Publication No. 86-23) and "Le Guide du Journal Officiel des Communautes Europeennes L358, 18 Decembre 1986, n° 86/609/CEE du 24 Novembre 1986".

Table KIIA 5.3.2/01-1: Study design

Group	No./sex/dose	Nominal dietary dose (ppm)	Achieved mean dose level (mg/kg bw/d)	
			M	F
1	10 + 10*	0	0	0
2	10	100	6.0	7.6
3	10	500	30.2	38.3
4	10 + 10*	2500	156	186

* These animals were sacrificed following a 30 or 31 day recovery period after termination of treatment. Data excerpted from pages 123 and 124 of the study report.

3. Diet preparation and analysis

   The required amount of the test material was incorporated into the diet by dry mixing to achieve the nominal dietary concentrations at each dosing level. Initially, there were two preparations (F1 and F2) for the study. For the highest

concentration of 2500 ppm, a second preparation (F2bis) was performed due to unacceptable results concerning the concentration of the second formulation of 2500 ppm (i.e. had achieved concentration of 82% of the nominal concentration). The first test substance formulation was prepared to cover the dietary requirements over approximately the first 4-weeks and the second and third formulations (F2 and F2bis) to cover the dietary needs until the end of the study. When not in use the diet formulations were stored at approximately -18°C.

The stability of BYI 02960 in the diet (concentrations of 20, 100 and 2500 ppm) was checked over a period of at least 92 or 81 days of frozen storage followed by 10 days at room temperature or for at least 102 or 91 days of storage at room temperature. BYI 02960 formulation at 2500 ppm was found to be stable in diet after at least 102 days of storage at room temperature and after 92 days of frozen storage followed by 10 days at room temperature. Samples of the 20 and 100 ppm preparations were found to be stable in diet after 81 and 92 days, respectively, of frozen storage followed by 10 days at room temperature. They were also found to be stable after at least 21 days of storage at room temperature.

The homogeneity of BYI 02960 in diet was verified on the first preparation at the lowest and highest concentrations to demonstrate adequate formulation procedures. Achieved homogeneity were in the range of 90% to 103% of the nominal concentration, with achieved concentrations overall in the range of 90% to 100% of the nominal concentration.

4. Statistics:

Statistical methods employed in this study are listed in Table KIIA 5.3.2/01-2.

Table KIIA 5.3.2/01-2. Statistics used in data analyses.

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant (p>0.05)	**If preliminary test is significant (p≤0.05)**
90 day sacrifice animals			
Terminal body weight, Bodyweight gain, Clinical chemistry, Exploratory locomotor activity, Grip strength, Select haematology parameters (*prothrombin time, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, %neutrophils, %lymphocytes, %reticulocytes*) Urinalysis (volume and refractive index), and Organ weight (absolute and relative)	Bartlett Test	Analysis of variance followed by 2-sided Dunnett's test if significant	Kruskal-Wallis test followed by 2-sided Dunn's test if significant
Body weight and mean food consumption per day parameters, and Select haematology parameters (*red blood cell count, platelet count, white blood cell count, neutrophil count, lymphocyte count, reticulocyte count)*	Bartlett Test	Analysis of variance followed by 2-sided Dunnett's test if significant.	Bartlett Test on Log transformed[a] data. If significant then Kruskal-Wallis test followed by 2-sided Dunn's test if significant result; Otherwise ANOVA followed by Dunnett test on transformed data if significant.
Urinary pH	Kruskal-Wallis test	No further analysis	2-sided Dunn's test
Recovery phase animals			
Terminal bodyweight Bodyweight gain Select haematology parameters (*prothrombin time, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, %neutrophils, %lymphocytes, %reticulocytes*) Clinical chemistry Quantitative urinalysis (volume and refractive index), Organ weight (absolute and relative)	F test	2-sided T-test	2-sided Modified T-test
Bodyweight and mean food consumption per day parameters Select haematology parameters (*red blood cell count, platelet count, white blood cell count, neutrophil count, lymphocyte count, reticulocyte count)*	F test	2-sided T-test	F-test on log transformed data[a], if significant then 2-sided Modified T-test; Otherwise 2-sided T-test on transformed data
Urinary pH	2-sided non-parametric Mann Whitney test		

[a] Except for haematology parameters, which were square root transformed.

C. METHODS

1. Observations

Animals were observed at least twice daily for mortality and morbidity (once daily on weekends/public holidays). Animals were observed for clinical signs at least once daily, and detailed physical examinations were undertaken weekly. Cage and cage trays were inspected daily for evidence of ill-health such as blood or loose faeces. Additionally, on study weeks 12 to 13, surviving animals were subjected to Functional Observational Battery (FOB) and exploratory locomotor activity assessment. The experimenter conducting the FOB was blind with respect to the treatment group of each animal. The parameters evaluated in the FOB are presented below.

Exploratory locomotor activity:

Rats were tested individually in automated photocell apparatus (Imetronic, Bordeaux, France) that recorded spontaneous exploratory locomotor activity in the novel environment of the apparatus over 90 minutes with data collected at intervals throughout this period.

Open field observations:

Posture
Bizarre behaviour (e.g. self-mutilation, walking backward)
Gait
Tremors (clonic) or seizures (tonic)
Stereotypic behaviour - e.g. excessive grooming, repetitive circling
Other neurological related changes

Sensory reactivity (in the standard arena):

Pupillary reflex
Corneal reflex
Surface righting reflex
Flexor reflex
Auditory startle response
Tail pinch response

Grip strength

The fore- and hindlimb grip strength were measured quantitatively using a grip strength tester.

2. Body weights

All animals were weighed at study initiation and at necropsy, and on a weekly basis over the dosing and recovery periods. Body weight change was calculated for each week and for the total study duration.

3. Food consumption, food efficiency and daily intake

Food consumption was recorded for each animal twice weekly for the first six weeks, then weekly thereafter. Daily intake was calculated from food

consumption and body weight data. Food use efficiency estimates were not undertaken.

4. Ophthalmological examinations

   All animals were examined by focal illumination and indirect ophthalmoscopy prior to study start. All surviving animals in the control and high dose group were examined again prior to scheduled sacrifice.

5. Clinical pathology (haematology, clinical chemistry, coagulation, urinalysis)

   Blood samples were collected for all animals by puncture of the retro-orbital plexus on study day 95, 96 or 97, or on recovery day 30 or 31. Animals were fasted overnight prior to bleeding and anesthetized by inhalation of isoflurane. Urine samples were collected on study days 90 or 91, or on recovery day 26. The following haematology, coagulation, clinical chemistry, and urinalysis parameters were evaluated on the samples. (Note: Blood smear examination was undertaken only if abnormal haematology results were obtained).

Hematology and coagulation

red blood cell count	hemoglobin
reticulocyte count	hematocrit
platelet count	mean corpuscular (cell) volume
white blood cell count	mean corpuscular hemoglobin
differential white blood cell count	
prothrombin time	
mean corpuscular (cell) hemoglobin concentration	
microscopic blood smear examination	

Clinical chemistry

aspartate aminotransferase	glucose
alanine aminotransferase	total protein
alkaline phosphatise	albumin
total bilirubin	calcium
creatinine	inorganic phosphorus
gamma glutamyltransferase	sodium
total cholesterol	urea
triglycerides	potassium
chloride	

Urinalysis

quality	ketone
volume	protein
specific gravity/osmolality/refractive index	bilirubin
pH	blood
glucose	urobilinogen
Microscopic urine sediment examination	

6. Plasma concentration

Blood (non-fasted) was collected from 5 males and 5 females of each dose group on study day 90, and immediately frozen at -20°C, for future determination of plasma concentration of test substance. Samples were evaluated by high performance liquid chromatography/tandem mass spectroscopy (HPLC-MS/MS).

7. Sacrifice and pathology

At termination (study day 95, 96 or 97, or recovery day 30 or 31), animals were sacrificed by exsanguination under deep anesthesia (inhalation of isoflurane) following an overnight fast. Gross examinations were performed on all animals. Organs that were weighed are listed in Table KIIA 5.3.2/01-3. Relative organ weight to body weight and to brain weight ratios were calculated. For those animals sacrificed on study day 95, 96 or 97 in the control and high dose groups (i.e. 2500 ppm) all the tissues (including gross lesions) indicated in Table KIIA 5.3.2/01-3 were collected and processed to slides and evaluated microscopically. For all other dose groups, only tissues from the liver, kidney, lung, and thyroid gland and gross lesions were processed and evaluated microscopically. For recovery group animals sacrificed on recovery day 30 or 31, only the liver, kidney, lung, thyroid gland and gross lesions were collected, and only the liver and thyroid gland were processed and evaluated microscopically.

Table KIIA 5.3.2/01-3: Organs/tissues collected for pathological examination

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted#
Adrenal gland	X	X	X
Aorta		X	X
Bone with bone marrow, sternum		X	X
Bone marrow smear^		X	
Brain	X	X	X
Epididymis	X	X	X
Esophagus		X	X
Eye (with optic nerve)		X	X
Harderian gland		X	X
Heart	X	X	X
Kidney	X	X	X
Intestines (duodenum, jejunum, ileum, cecum, colon, rectum)		X	X
Lacrymal exorbital gland		X	
Larynx/pharynx		X	
Liver	X	X	X
Lung		X	X
Lymph nodes (submaxillary, mesenteric)		X	X
Mammary gland		X	X

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted#
Nasal cavities		X	
Ovary	X	X	X
Pancreas		X	X
Parathyroid gland		X	X
Pituitary	X	X	X
Prostate	X	X	X
Salivary gland, submaxillary		X	X
Sciatic nerve		X	X
Skeletal muscle		X	X
Seminal vesicle		X	X
Skin		X	X
Spinal cord, (cervical, lumbar, thoracic)		X	X
Spleen	X	X	X
Stomach		X	X
Testis	X	X	X
Thymus	X	X	X
Thyroid gland	X	X	X
Tongue		X	X
Trachea		X	X
Urinary bladder		X	X
Uterus with cervix	X	X	X
Vagina		X	X
Gross lesions		X	X

For animals in the low and mid dose groups (i.e. 100 and 500 ppm) in the main study, only tissues from the liver, kidney, lung, and thyroid along with gross lesions were processed to slides and examined microscopically. For animals in the recovery groups, only the liver and thyroid gland were processed to slides and examined microscopically.

^ A bone marrow smear of one femur was prepared for each animal, but was not examined for any group due to the absence of abnormal haematology findings.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity/General findings

   No test substance-related clinical signs were observed during the treatment and recovery periods.

2. Mortality

   There was no unscheduled mortality.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 2500 ppm compared to controls, a statistically significant lower body weight was observed in males (6-7%) and females (6-10%) throughout the main study, with the exception of day 92 in males when the observed decrease was not statistically significant (Tables KIIA 5.3.2/01-4). At study termination a decrease of 6% (statistically significant in females only) was seen in both sexes. Additionally at 2500 ppm, statistically significant decreases in cumulative mean body weight gain were seen throughout the study in both sexes. At study termination, a decrease of 12% and 15% was seen for cumulative body weight gain in males and females respectively (KIIA 5.3.2/01-5).

At 500 ppm compared to controls, throughout the study minimal decreases in body weight of 1% to 3% were seen in males, with decreases of 2% to 5% seen in females throughout the study. None of these findings in either sex was statistically significant. However, the overall mean body weight gain at this dose was 12% lower ($p \leq 0.05$) than the controls in females by the end of the treatment period (Study Day 92).

At 100 ppm, body weight or body weight gain in either sex was not affected.

Table KIIA 5.3.2/01-4. Body weights (g) in the main groups

Study day	Group mean bodyweight (grams)							
	M				F			
	Dose (ppm)							
	0	100	500	2500	0	100	500	2500
1	220±8	220±7 (0%)	218±6 (-1%)	219±7 (0%)	174±6	174±7 (0%)	174±6 (0%)	172±8 (-1%)
8	280±12	280±12 (0%)	274±6 (-2%)	**260±11 (-7%)****	199±9	200±10 (1%)	194±6 (-3%)	**180±8 (-10%)****
15	328±19	332±21 (1%)	323±9 (-2%)	**308±17 (-6%)****	219±8	221±15 (1%)	212±6 (-3%)	**202±10 (-8%)****
22	365±23	372±28 (2%)	361±14 (-1%)	**342±22 (-6%)****	233±10	238±16 (2%)	225±12 (-3%)	**217±12 (-7%)****
29	393±27	400±33 (2%)	389±16 (-1%)	**368±24 (-6%)****	244±10	249±17 (2%)	238±14 (-2%)	**229±13 (-6%)****
36	419±30	429±37 (2%)	408±27 (-3%)	**391±28 (-7%)***	250±10	258±20 (3%)	242±12 (-3%)	**236±14 (-6%)****
43	435±36	447±40 (3%)	425±25 (-2%)	**404±28 (-7%)***	260±10	262±19 (1%)	252±10 (-3%)	**243±14 (-7%)****
50	452±40	464±42 (3%)	445±28 (-2%)	**422±30 (-7%)***	266±12	270±17 (2%)	256±12 (-4%)	**248±14 (-7%)****
57	470±39	485±46 (3%)	463±30 (-1%)	**436±32 (-7%)***	271±10	277±19 (2%)	260±10 (-4%)	**254±13 (-6%)****
64	482±42	497±49 (3%)	479±35 (-1%)	**448±35 (-7%)***	275±11	279±21 (1%)	263±8 (-4%)	**257±13 (-7%)****
71	491±43	509±53 (4%)	487±35 (-1%)	**457±37 (-7%)***	278±10	282±22 (1%)	266±11 (-4%)	**260±14 (-6%)****
78	502±44	519±56 (3%)	497±35 (-1%)	**467±39 (-7%)***	280±11	284±22 (1%)	270±14 (-4%)	**267±14 (-5%)****

Study day	Group mean bodyweight (grams)							
	M				F			
	Dose (ppm)							
	0	100	500	2500	0	100	500	2500
85	509±45	525±58 (3%)	506±36 (-1%)	**475±41 (-7%)***	280±12	288±20 (3%)	273±11 (-3%)	**266±14 (-5%)****
92	512±44	528±59 (3%)	505±39 (-1%)	479±41 (-6%)	284±14	284±18 (0%)	271±11 (-5%)	**266±14 (-6%)****

* Denotes that the group mean is significantly different from the control at p ≤0.05 or **p ≤0.01 by Dunnett's test. Data excerpted from pages 89 to 92 of the study report.

Table KIIA 5.3.2/01-5: Mean Cumulative body weight gain (g)

Study day interval	M				F			
	Dose (ppm)							
	0	100	500	2500	0	100	500	2500
1 to 8	60±6	60±8 (0%)	57±3 (-5%)	**41±7 (-32%)****	25±5	26±7 (4%)	**20±4 (-20%)***	**7±5 (-72%)****
9 to 15	108±13	113±16 (5%)	106±9 (-2%)	**89±13 (-18%)****	45±5	47±10 (4%)	38±5 (-16%)	**30±6 (-33%)****
16 to 22	146±17	152±24 (4%)	143±15 (-2%)	**122±19 (-16%)****	59±7	64±11 (8%)	51±9 (-14%)	**45±8 (-24%)****
23 to 29	174±22	180±29 (3%)	171±18 (-2%)	**149±21 (-14%)****	70±6	75±13 (7%)	64±10 (-9%)	**56±9 (-20%)****
30 to 36	200±24	209±33 (5%)	191±29 (-5%)	**171±26 (-15%)****	76±9	84±17 (11%)	68±10 (-11%)	**64±11 (-16%)****
37 to 43	216±31	227±36 (5%)	208±27 (-4%)	**185±26 (-14%)****	86±8	87±16 (1%)	78±8 (-9%)	**71±10 (-17%)****
44 to 50	232±34	245±39 (6%)	228±30 (-2%)	**202±28 (-13%)***	92±9	96±13 (4%)	**82±10 (-11%)***	**76±10 (-17%)****
51 to 57	251±33	266±43 (6%)	245±32 (-2%)	**216±29 (-14%)****	97±8	102±15 (5%)	**86±8 (-11%)***	**81±10 (-16%)****
58 to 64	263±37	277±46 (5%)	261±37 (-1%)	**229±32 (-13%)***	101±10	104±17 (3%)	**89±11 (-12%)***	**85±10 (-16%)****
65 to 71	271±37	289±50 (7%)	270±37 (0%)	**238±34 (-12%)***	105±9	108±18 (3%)	**92±11 (-12%)***	**87±11 (-17%)****
72 to 78	283±38	299±53 (6%)	279±37 (-1%)	**248±37 (-12%)***	106±9	110±19 (4%)	96±12 (-9%)	**94±11 (-11%)****
79 to 85	290±40	305±56 (5%)	289±38 (0%)	**256±39 (-12%)***	106±9	113±16 (7%)	99±11 (-7%)	**93±10 (-12%)****
86 to 92	293±40	308±57 (5%)	287±41 (-2%)	**259±40 (-12%)***	110±12	110±15 (0%)	**97±9 (-12%)***	**94±11 (-15%)****

* denotes values that are statistically significant at the * p≤0.05 or ** p≤0.01 level.
Data excerpted from pages 107 to 113 of the study report.

In recovery group animals, body weight differences associated with treatment at 2500 ppm were reversible, as mean body weight approached control levels by the end of the recovery period (Table KIIA 5.3.2/01-6).

Table KIIA 5.3.2/01-6: Bodyweights (g) in recovery group phase

	Group mean bodyweight (±SD) and per cent change from control	
	M	F

	Dose (ppm)			
Study day	**0**	**2500**	**0**	**2500**
1	511±38	476±49 (-7%)	290±14	**272±13 (-6%)****
8	518±40	496±55 (-4%)	290±13	284±15 (-2%)
15	522±40	507±57 (-3%)	294±12	284±17 (-3%)
22	529±43	512±57 (-3%)	294±13	284±13 (-3%)
29	527±43	514±59 (-2%)	297±13	290±15 (-2%)

** Denotes that the group mean is significantly different from the control at p ≤0.01 by Dunnett's test. No changes in recovery group males were statistically significant (p≤0.05). Data excerpted from pages 94 and 95 of the study report.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

At 2500 ppm, a slight statistically significant reduction in mean food consumption (17%, p≤0.01) was observed for males during the first four days of the study and thereafter on several occasions (~5%; p≤ 0.05) from Study Day 71 until the end of the dosing phase. In females, a reduction in mean food consumption was observed from the first week of the study until week 7 (between 9% and 29%). Thereafter, decreased mean food consumption was observed (-4 to -10%) without statistical significance (Table KIIA 5.3.2/01-7).

No treatment-related effects on mean food consumption were observed for both sexes at 100 or 500 ppm.

	Table KIIA 5.3.2/01-7 Mean food consumption/day (g/day)							
	M				**F**			
	Dose (ppm)							
Study day	**0**	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
4	24.1±0.6	23.4±0.2 (-3%)	23.4±0.7 (-3%)	**20.1±1.2 (-17%)****	18.2±0.2	17.9±1.2 (-2%)	17±0.7 (-7%)	**13±0.9 (-29%)****
8	25.4±0.4	25±0.3 (-2%)	24.1±1.1 (-5%)	24.8±1.3 (-2%)	18.9±0.4	19.5±1.2 (3%)	17.2±0.1 (-9%)	**16.2±1.6 (-14%)***
11	26.7±0.7	26.4±0.5 (-1%)	25.8±1.1 (-3%)	25.8±0.4 (-3%)	19.7±0.7	20.2±0.8 (3%)	18.5±1.1 (-6%)	**17.4±0.6 (-12%)****
15	26.7±0.6	27.4±0.5 (3%)	27.5±0.9 (3%)	26.8±1.1 (0%)	19.3±0.3	19.9±1.4 (3%)	19.1±1 (-1%)	18.8±1.3 (-3%)
18	26±0.7	26.4±0.1 (2%)	26.3±1.9 (1%)	25.3±0.4 (-3%)	19.2±0.2	19.5±2.4 (2%)	17.3±0.3 (-10%)	**16.9±0.3 (-12%)***
22	26±0.2	26.6±0.1 (2%)	25.6±1.1 (-2%)	25.5±0.6 (-2%)	19.3±0.3	18.9±1.3 (-2%)	18±0.1 (-7%)	**16.9±0.4 (-12%)****
25	26.4±0.6	26.2±0.5 (-1%)	25.4±0.8 (-4%)	25.6±0.4 (-3%)	19.8±0.9	19.7±1.6 (-1%)	18.4±0.2 (-7%)	**17.2±0.3 (-13%)****
29	25.9±0.5	25.7±0.4 (-1%)	25.3±1.1 (-2%)	25.5±0.6 (-2%)	19.8±0.3	19.4±0.8 (-2%)	19±0.3 (-4%)	**16.9±0.3 (-15%)****
32	26.4±0.6	26.9±0.9 (2%)	26±0.8 (-2%)	**25±0.6 (-5%)***	19.3±0.7	19.4±1.7 (1%)	19±0.8 (-2%)	**17±0.4 (-12%)***

Study day	Table KIIA 5.3.2/01-7 Mean food consumption/day (g/day)							
	M				F			
	Dose (ppm)							
	0	100	500	2500	0	100	500	2500
36	25.7±0.4	25.7±0.1 (0%)	24.4±1.8 (-5%)	24.8±0.4 (-4%)	19.1±0.6	19±1.4 (-1%)	18.2±0.5 (-5%)	**17.1±0.4 (-10%)****
39	25.1±0.3	25.5±0.2 (2%)	24.3±1.3 (-3%)	24.9±0.7 (-1%)	20.3±1	18.9±1 (-7%)	19.3±0.9 (-5%)	**17.7±0.7 (-13%)****
43	25.4±1.4	26.5±0.1 (4%)	24.6±0.5 (-3%)	25±0.3 (-2%)	20.1±0.5	19.8±1.2 (-1%)	18.9±0.7 (-6%)	**17.5±0.2 (-13%)****
50	25.7±1	26.8±0.1 (4%)	25.5±1 (-1%)	25.1±0.4 (-2%)	19.6±0.2	19.7±1.3 (1%)	18.4±0.1 (-6%)	**17.8±0.7 (-9%)***
57	25.5±0.9	26.4±0.5 (4%)	25.8±0.8 (1%)	24.5±0.3 (-4%)	19.1±0.2	19.9±1.8 (4%)	18.4±0.6 (-4%)	18±0.9 (-6%)
64	25.1±0.6	25.8±0.1 (3%)	25.5±0.5 (2%)	24.3±0.5 (-3%)	19.3±0.9	19.8±2.2 (3%)	18.1±0.9 (-6%)	18.5±0.9 (-4%)
71	24.6±0.4	25.6±0.3 (4%)	25.1±0.9 (2%)	**23.4±0.4 (-5%)***	19.4±0.5	20.8±0.9 (7%)	19.7±2.1 (2%)	17.9±1 (-8%)
78	24.5±0.2	24.9±0.3 (2%)	24.7±0.8 (1%)	**23.3±0.7 (-5%)***	18.5±0.1	17.8±1.5 (-4%)	18±0.1 (-3%)	17.1±0.9 (-8%)
85	24.6±0.2	24.5±0.5 (0%)	24.5±0.3 (0%)	**23.4±0.3 (-5%)****	18.8±0.5	18.8±0.8 (0%)	18.5±0.2 (-2%)	17.4±0.8 (-7%)
92	23.8±1.3	22.5±0.4 (-5%)	22±1 (-8%)	22.1±1.4 (-7%)	17.8±1.2	15.8±0.2 (-11%)	16.3±0.3 (-8%)	16±1.4 (-10%)

* Denotes that the group mean is significantly different from the control at p ≤0.05 or **p ≤0.01.
Data excerpted from pages 116 to 119 of the study report.

No treatment-related changes were observed in food consumption in 2500ppm animals during the recovery period.

D. OPHTHALMOLOGICAL EXAMINATIONS

Ophthalmological findings included haemorrhage of the iris and a damaged eye noted in one male (ST4M0480) and one female (ST4F0499) at 2500 ppm. The study authors regarded that as single occurrences, the findings were unrelated to the test substance. The evaluator agreed with this conclusion.

E. CLINICAL PATHOLOGY

1. Hematology

In females at 2500 ppm, mean platelet counts were 15% higher (≤0.05) than controls, whereas group mean values for this parameter were unremarkable in all other test substance groups (Table KIIA 5.3.2/01-8). Mean white blood cell counts were also elevated by 39% in females at 2500 ppm when compared to controls, although statistical significance was not attained. These parameters were not affected in animals from the 2500 ppm dose group after the 28-day recovery period.

Table KIIA 5.3.2/01-8: Hematological findings (main study)

	Dosing (ppm)							
	M				F			
N=10	0	100	500	2500	0	100	500	2500
Platelet count (10^9/L)	1160±168	1121±193	1224±130	1235±121	1233±130	1324±143	1187±149	1415±203*
		(-3%)	**(+6%)**	**(+6%)**		**(+7%)**	**(-4%)**	**(+15%)**
WBC count (10^9/L)	13.1±2.9	12.9±2.1	13.7±2.4	14.7±2.9	8.8±1.6	7.4±2.2	7.3±1.7	12.2±4.4
		(-2%)	**(+5%)**	**(+12%)**		**(-16%)**	**(-17%)**	**(+39%)**

* Significantly different from the control at $p \leq 0.05$ by Dunnett's test. No haematology findings in males were statistically significant (i.e., p was >0.05). Data excerpted from pages 139, 140, 143 and 144 of the study report.

2. Clinical chemistry

Statistically significant ($p \leq 0.05$) differences clinical chemistry parameters were confined to the 2500 ppm dosing groups. In both sexes at this dose, lower total bilirubin (38/45% lower M/F, $p \leq 0.01$) and glucose (21/22% lower M/F, $p \leq 0.1$), and increased total cholesterol (28/46% increased, $p \leq 0.01$ in F only) and triglycerides (35/66% M/F, not statistically significant) were observed relative to controls (Table KIIA 5.3.2/01-9).

Additional differences not described by the study author occurred in females at 2500 ppm, with statistically significant ($p \leq 0.05$ or 0.01) increases in potassium, calcium, phosphorous, and decreased chlorine levels (data not shown except for phosphorus levels) relative to controls. However, changes in these parameters were small and considered to be within expected inter-animal variability.

Overall, the study authors regarded these clinical chemistry changes to be test substance related but reversible, except for total bilirubin in females at 2500 ppm. The evaluator also notes that statistically significantly ($p \leq 0.05$) higher phosphorous levels remained in females at 2500 ppm after the recovery period. However, while considered a treatment-related effect, the increased phosphorous levels in females are not expected to be toxicologically significant in this case.

Table KIIA 5.3.2/01-9: Clinical chemistry findings (main study)

	Dosing (ppm)							
	M				F			
	0	100	500	2500	0	100	500	2500
Main study								
Total bilirubin (µmol/L)	1.3±0.3	1.1±0.3 (-15%)	1.1±0.2 (-15%)	0.8±0.2** (-38%)	2.0±0.5	1.9±0.5 (-5%)	1.8±0.4 (-10%)	1.1±0.3** (-45%)
Glucose (mmol/L)	6.59±1.10	6.56±0.58 0%	6.19±0.91 (-6%)	5.23±0.40** (-21%)	6.03±0.78	5.82±0.97 (-3%)	5.58±0.46 (-7%)	4.69±0.54** (-22%)
Total cholesterol (mmol/L)	1.54±0.33	1.78±0.23 (+16%)	1.70±0.16 (+10%)	1.97±0.44 (+28%)	1.55±0.24	1.68±0.35 (+8%)	1.86±0.37 (+20%)	2.26±0.28** (+46%)
Triglycerides (mmol/L)	0.85±0.32	1.03±0.36 +21%)	0.85±0.39 0%	1.15±0.48 (+35%)	0.44±0.12	0.44±0.26 0%	0.48±0.13 (+9%)	0.73±0.32 (+66%)
Phos (mmol/L)	1.98±0.13	1.83±0.20 (-8%)	1.93±0.18 (-3%)	2.03±0.13 (+3%)	1.51±0.12	1.48±0.20 (-2%)	1.50±0.18 (-1%)	1.71±0.15* (+13%)
Recovery Study								
Phos (mmol/L)	1.81±0.12	**NA**	NA	1.82±0.12 (0%)	1.39±0.14	**NA**	NA	1.55±0.16* (+10%)
Total bilirubin (µmol/L)	1.2±0.5	NA	NA	1.3±0.2 (+8%)	2.4±0.7	NA	NA	1.8±0.5* (-25%)

Statistically significant from control at *p≤0.05 or ** p≤0.01. 'NA' – 'not available', as the recovery group did not include 100 or 500 ppm dose groups. Data excerpted from pages 63, 158, 160-162 and 165–166 of the study report, with the exception of per cent change from control for phosphorous levels and for parameters in recovery animals, which were calculated by the evaluator using Microsoft Excel.

3. Urinalysis

Treatment-related urinalysis findings were confined to an increasing trend for overnight urinary volumes (Table KIIA 5.3.2/01-10) in both sexes (14/75% increases for M/F) at 2500 ppm relative to controls, but without dose dependence or statistical significance in either sex (i.e., p >0.05). This finding was not observed in recovery group females, but remained present in recovery group males (30% increase, not statistically significant; Table KIIA 5.3.2/01-11). The urinary volume data were not discussed by the study author. Noting the lack of a consistent response in this parameter, the lack of statistical significance in the parameter, the large standard deviations (particularly in 2500 ppm females, in which a volume of 9.7 mL was recorded for one female versus 2.0 to 3.7 mL in the remaining animals in this dose group), and lack of treatment-related effects in urinalysis and renal histopathology, the evaluator considers the change in urinary volume as unlikely to be related to treatment, and attributable to individual animal variation.

Table KIIA 5.3.2/01-10: Overnight urinary volumes on study days 90/91

	Dosing (ppm)							
	M				F			
	0	100	500	2500	0	100	500	2500
Urinary vol (mL)	4.9±1.1	4.4±1.6 (-10%)	5.2±1.8 (+6%)	5.6±1.7 (+14%)	2.0±1.2	2.6±0.9 (+30%)	2.1±1.5 (+5%)	3.5±2.4 (+75%)

Data excerpted from pages 169 and 170 of the study report, except for per cent change from control, which was calculated by the evaluator.

Table KIIA 5.3.2/01-11: Overnight urinary volumes on recovery day 28

	Dosing (ppm)			
	M		F	
N=10	0	2500	0	2500
Urinary vol (mL)	4.3±1.5	5.6±2.5 (+30%)	2.7±0.6	2.3±1.0 (-15%)

Data excerpted from pages 176 and 177 of the study report, except for per cent change from control, which was calculated by the evaluator.

F. PLASMA CONCENTRATIONS OF TEST MATERIAL

At day 90/91, the concentrations of test substance in plasma were higher in females than in males at all concentrations of test substance in feed, with a 1.3-fold, 1.4-fold, and 1.7-fold higher concentration of test material at 100, 500, and 2500 ppm, respectively (Table KIIA 5.3.2/01-12). While dose-linearity was observed in plasma test material concentrations between 100 and 500 ppm in both sexes (4.9/5.5× increases for M/F respectively), sub-linear increases in plasma test material concentration were noted between 100 and 500 ppm (15.4/21.3× increases for M/F respectively).

Table KIIA 5.3.2/01-12: Individual & group mean plasma concentrations of BYI 02960 (mg/L±SD)

N=5	Dose (ppm)			
	0	100	500	2500
Males				
Animal Nº's [(B)]	**#0390-0394**	**0430 to 0434**	**0450 to 0454**	**0470 to 0474**
	<LLOQ	1.40	5.36	16.9
	<LLOQ	1.46	8.31	24.4
	<LLOQ	1.39	6.13	25.8
	0.032	1.52	8.14	19.0
	<LLOQ	1.50	7.40	25.3
Group Mean	<LLOQ	1.45±0.06	7.07±1.28	22.3±4.05
Fold-increase from 100 ppm	**-**	**1**	**4.9**	**15.4**
Females				
Animal Nº's	**0410 to 0414**	**0440 to 0444**	**0460 to 0464**	**0490 to 0494**
	<LLOQ	1.34	12.2	46.5
	<LLOQ	1.79	7.41	31.3
	<LLOQ	1.92	9.21	36.9
	<LLOQ	2.02	9.93	31.9
	<LLOQ	2.09	11.4	48.4
Group Mean	<LLOQ	1.83±0.30	10.0±1.88	39.0±8.04

N=5	Dose (ppm)			
	0	100	500	2500
Males				
Fold-increase from 100 ppm	-	**1**	**5.5**	**21.3**
Female:male test material ratios in plasma	-	***1.3***	***1.4***	***1.7***

Lower limit of quantification (LLOQ) = 0.025 mg/L. 'B' – the first four characters in each animal number have been omitted in the above table but are presented in full elsewhere in this study evaluation. Data excerpted from pages 14 and 15 of 663, except for fold-increases from 100 ppm values and fold increase from males, which were calculated by the evaluator using Microsoft Excel.

G. FUNCTIONAL OBSERVATIONAL BATTERY (FOB)

No treatment-related changes were observed in the FOB.

H. SACRIFICE AND PATHOLOGY

1. Organ weight

Organ weight findings were confined to increases in the absolute and relative (to bodyweight and brain) weights of the liver and thyroid in both sexes (Table KIIA 5.3.2/01-13).

Absolute liver weight were increased by 8% in both sexes at 2500 ppm relative to controls (not statistically significant), and absolute thyroid weights were increased by 20%/6% in M/F (not statistically significant). When expressed as relative-to-brain weight ratios, similar magnitude of changes were observed in liver and thyroid in both sexes.

Statistically significant relative to bodyweight increases in liver weight ratios (16/15% for M/F, $p \leq 0.01$) at 2500 ppm, and thyroid weight ratios in males at $\geq$ 500 ppm (20–26% increases, $p \leq 0.05$) were noted.

All differences in liver and thyroid weights between controls and 2500 ppm recovery groups had reversed by the end of the recovery period (data not shown).

Both of the increased relative (to bodyweight) weights of the liver in both sexes, and the thyroid in males at 2500 ppm were regarded by the study authors to be test substance related on the basis that were accompanied by histopathological findings in these organs. However, noting the lack of gross and histopathological effects at 500 ppm, the change in organ weight for 500 ppm male animals is not considered a toxicologically adverse effect in this case.

Table KIIA 5.3.2/01-13: Organ weights data from rats administered BYI 02960 for 90 days

Organ parameter	M				F			
	Dose (ppm)							
	0	100	500	2500	0	100	500	2500
Terminal body weight (g)	497.4 ±49.5	508.1 ±57.8 (+2%)	484.5 ±38.8 (-3%)	460.7 ±32.3 (-7%)	270.0 ±11.7	272.2 ±19.4 (+1%)	259.9 ±9.9 (-4%)	252.8* ±14.8 (-6%)
Absolute liver weight (g)	10.92 ±1.44	11.37 ±1.85 (+4%)	10.92 ±1.01 (0%)	11.78 ±1.43 (+8%)	6.18 ±0.27	5.91 ±0.58 (-4%)	5.93 ±0.29 (-4%)	6.65 ±0.82 (+8%)
Liver to body weight ratio (%)	2.194 ±0.164	2.231 ±0.172 (+2%)	2.255 ±0.155 (+3%)	**2.546** **±0.200 (+16%)**	2.29 ±0.115	2.17 ±0.095 (-5%)	2.283 ±0.086 (0%)	**2.624** **±0.232 (+15%)**
Liver to brain weight ratio (%)	504.824 ±63.640	527.855 ±86.107 (+5%)	494.455 ±52.605 (-2%)	546.074 ±65.306 (+8%)	303.728 ±16.528	290.22 ±29.691 (-4%)	294.529 ±11.795 (-3%)	333.353 ±42.388 (+10%)
Absolute thyroid weight (g)	0.0195 ±0.0028	0.0222 ±0.0031 (+14%)	0.0228 ±0.0027 (+17%)	0.0234 ±0.0046 (+20%)	0.0162 ±0.0023	0.0162 ±0.0017 (0%)	0.0165 ±0.0026 (+2%)	0.0171 ±0.0028 (+6%)
Thyroid to body weight ratio (%)	0.00393 ±0.00042	0.00439 ±0.00055 (+12%)	**0.00473*** **±0.00068 (+20%)**	**0.00494**** **±0.00078 (+26%)**	0.00602 ±0.00095	0.00599 ±0.00095 (0%)	0.00633 ±0.00095 (+5%)	0.00679 ±0.00108 (+13%)
Thyroid to brain weight ratio (%)	0.9033 ±0.12280	1.02835 ±0.12300 (+14%)	1.0295 ±0.11047 (+14%)	1.08348 ±0.21085 (+20%)	0.7992 ±0.12971	0.7939 ±0.09540 (-1%)	0.81569 ±0.11771 (+2%)	0.85994 ±0.14222 (+8%)

* denotes statistical significance at the $p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from page 65 of the study report.

2. Gross pathology and histopathology

In gross pathology findings, hepatomegaly was observed in 4/10 males and 1/10 females at 2500 ppm (no incidence in other groups; Table KIIA 5.3.2/01-14). This finding was correlated to hepatocellular hypertrophy present in all 2500 ppm males at a minimal (N=6) or slight (N=4) severity and in 3/10 females at 2500 ppm with minimal severity. Hepatocellular hypertrophy was regarded by the study authors to be test substance related. Additionally, prominent lobulation of the liver was observed in one of the four males at 2500 ppm presenting with hepatomegaly and hepatocellular hypertrophy. Liver lobulation was not discussed by the study authors, but was seen in the 28 day BYI 02960 dosing study in rats in association with hepatocellular hypertrophy (Capt, 2009a: SA 06075). However, the US EPA secondary reviewer considered the liver effect to be adaptive response as it did not have any correlated clinical chemistry or histological changes.

Follicular cell hypertrophy of minimal severity was present in 3/10 males at 2500 ppm, but was not present in any other groups including controls. In one of the affected males, the thyroid also had a dark discoloration. The study authors regarded both findings in the thyroid as being test substance related.

Histopathological findings in the recovery groups at 2500 ppm were unremarkable (data not shown), and therefore the hepatocellular hypertrophy, lobulation, and follicular cell hypertrophy were considered readily reversible.

Table KIIA 5.3.2/01-14: Necropsy and histological findings in main dosing group animals (Study day 92 or 93)

	Dose (ppm)							
	Males				Females			
	0	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
Gross necropsy findings								
Hepatomegaly								
Total	0/10	0/10	0/10	4/10	0/10	0/10	0/10	1/10
Prominent lobulation								
Total	0/10	0/10	0/10	1/10	0/10	0/10	0/10	0/10
Histopathological findings								
Diffuse centrilobular hepatocellular hypertrophy								
Minimal	0	0	0	6	0	0	0	3
Slight	0	0	0	4	0	0	0	0
Total	0/10	0/10	0/10	10/10	0/10	0/10	0/10	3/10
Diffuse follicular cell hypertrophy (minimal severity)								
Total	0/10	0/10	0/10	3/10	0/10	0/10	0/10	0/10

Statistical analysis not undertaken. Data excerpted from pages 66 and 67 of the study report.

III. CONCLUSION

Under the conditions of this study, BYI 02960 produced no effects on clinical signs or changes in FOB parameters, unscheduled mortality, or ophthalmology.

In haematological examinations, mean platelet counts were 15% higher (≤0.05) than controls in 2500 ppm females, though this finding was reversible 30 days after cessation of dosing. While clinical chemistry analyses reported a variety of adaptive changes in bilirubin, glucose, cholesterol, triglycerides, potassium, calcium and chloride, the reported changes were not considered toxicologically adverse.

The study also examined test substance concentrations in plasma during the last week of the study; the results indicated a linear increasing trend between plasma concentration vs dose level administered between 100 and 500 ppm, but a sub-linear increasing trend between 100 and 2500 ppm plasma concentrations of test substance. A slight sex-specific difference in test substance plasma concentrations was noted, with higher levels observed in females (1.3–1.7× higher than males).

At 500 ppm, there was a 12% reduction in mean overall bodyweight gain in females relative to controls. In the absence of other toxicologically adverse findings this decrease was not adverse. Thus, no compound-related adverse effects were seen at 100 and 500 ppm.

At 2500 ppm, decreases in mean absolute body weight and body weight gains were observed in both sexes during the treatment period. During day 8 measuring interval, the absolute body weight decrement in females reached 10%, and at day 14 the decrease was 8%. Thereafter, the decrease in body weight was below 8% and persisted until the end of the study. The slight body changes were not considered adverse in males; for females, the decrease (↓10%) reached the magnitude of being adverse at the beginning of the study. The adverse body weight effect in females might reflect higher concentrations of the test compound in females relative to males. The food consumption was consistently decreased in both males and females (↓9% to ↓29%). The increased incidence of gross and histopathological changes in the thyroid (dark color thyroid and thyroid follicular cell hypertrophy) combined with increased absolute and relative thyroid weights are considered to be adverse.

Therefore, the NOAEL in both sexes was 500 ppm (38 mg/kg bw/day); LOAEL was 2500 ppm (156 mg/kg bw/day) based on increased incidence of thyroid follicular cell hypertrophy with associated increases of absolute and relative thyroid weights, decreased body weights in females, and decreased food consumption in both sexes.

This study is classified as fully reliable (acceptable/guideline) and satisfies guideline requirements for a subchronic oral toxicity in rats [OPPTS 870.3100; OECD 408].

Primary review: Australia
Secondary review: US EPA & PMRA

Report: Odin-Feurtet M (2012) BYI 0260. 90-day toxicity study in the mouse by dietary administration. Bayer SAS. Bayer CropScience. 355 rue Dostoïevski. BP 153, 06903 Sophia Antipolic Cedex, France. Laboratory Report No.: SA 07295. March. MRID 48844112. PMRA 2239436. Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N°TXRVP009. Bayer CropScience File No. M-328668-03-2. Report Amendment Nº 2 date: 22 March 2012. Report Amendment Nº 1 date: 27 July 2011. Original report date: 6 Febuary 2009.

Guidelines: OPPTS 870.3100 (1998)
OECD 408 (1998)
Commission Directive 2001/59/EC Part B.26 (2001)
MAFF 12 Nousan 8147 (2000)

Deviations: None

GLP: Yes. Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a 90-day dietary study (MRID 48844112), BYI 02960 (99.5%; NLL 7780-44-6) was administered to six week old C57BL/6J mice in groups of 10/sex/dose at 0, 100, 500, or 2500 ppm (equivalent to 0/0, 15.6/18.8, 80.7/98.2, or 407/473 mg/kg bw/d). Analysis of the plasma concentration of the test substance was conducted on blood samples of 5/sex/dose group.

Under the conditions of this study BYI 02960 did not affect mortality or produce clinical signs of toxicity. In addition, the test chemical did not produce adverse effects at 100 and 500 ppm.

In males at 2500 ppm, lower bodyweight gain led to terminal bodyweights being 10% lower than controls. While decreases in bodyweight gain in males may be associated with decreased absolute food consumption, particularly in study weeks 1 to 2, where up to 11% lower consumption was observed Terminal bodyweights were unaffected in 2500 ppm females when accounting for differences in starting bodyweights.

at 2500 ppm, clinical chemistry examinations showed a 30/24% (M/F, $p \leq 0.01$) decrease in serum cholesterol, 38% ($p<0.01$) increase in alkaline phosphatase (ALP) activity in male serum, and 106% increase in alanine aminotransferase (ALT) activity in female serum.. Absolute and relative liver weights were increased in females at 2500 ppm. When the clinical chemistry parameter changes were considered in the context of an increased severity of diffuse centrilobular hepatocellular hypertrophy in both sexes, these findings were consistent with toxicologically adverse hepatocellular toxicity in both sexes.

Additionally at 2500 ppm, in clinical chemistry examinations there were increases of 51% in serum urea ($p \leq 0.05$) in males and a 5% decrease ($p \leq 0.05$) in serum protein in both sexes. When considered in the context of an 11% ($p < 0.01$) decrease in absolute and relative (to brain weight) kidney weight, along with the absence of multifocal/diffuse cortical epithelial cell vacuolation, collectively, these changes suggest test substance related, toxicologically adverse renal toxicity in males at 2500 ppm.

The NOAEL was 500 ppm (81 mg/kg/day) for both sexes. The LOAEL was 2500 ppm (407 mg/kg/day) based on based on consistent decreases in body weight (>10%) with associated reduction in body weight gains in male, liver effects (↑ ALP and ALT, ↓ in total cholesterol, ↑ in absolute and relative liver weights, increased incidence of moderate diffuse hepatocellular hypertrophy). Kidney effects (changes in clinical chemistry parameters and loss of multifocal/diffuse cortical epithelial cell vacuolation & increases in urea).

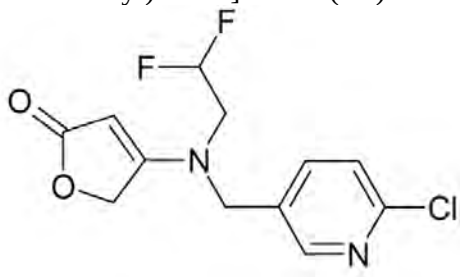
The results of the plasma analysis showed that test substance concentrations in plasma at 90-days were higher in males than in females at all dose levels. In both sexes, a generally linear correlation between administered concentrations of test substance and plasma concentrations was observed.

This study is classified as fully reliable (acceptable/guideline) and satisfies guideline requirements for a subchronic oral toxicity in mice [OPPTS 870.3100; OECD 408].

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one



Lot/Batch #:	NLL 7780-44-6
Purity:	99.5%
Description:	Pink powder
CAS #	Not reported
Stability of test compound:	Stated to be at least six months if stored as recommended at 25±5ºC in an air tight, light resistant container (date of analysis 16 Jan 2008). Achieved concentration and homogeneity analyses in prepared diet were undertaken, but stability analysis after storage or in animal rooms was not undertaken.

2. Vehicle and/or positive control: Untreated diet. No positive control in this study.
3. Test animals

Species:	Mouse
Strain:	C57BL/6J
Age at dosing:	6 weeks old
Weight at dosing:	18.9–21.9 g for males; 12.9–17.9 g for females
Source:	Charles River Laboratories, St. Germain-sur-l'Arbresle, France
Acclimation period:	12 days
Diet:	A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*.
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Three per sex and per cage from arrival to pre-study Day 5, then individually thereafter. Housed in suspended, stainless steel wire mesh cages.

4. Environmental conditions

Temperature:	20 to 24°C
Humidity:	40 to 70%
Air changes:	10 to 15/hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion

28 February 2008 to 19 September 2008. Blood samples analysis for concentration of BYI 02960 in plasma undertaken 5–20 October 2011.

2. Animal assignment and treatment

The dosing regimen of 0, 100, 500, or 2500 ppm was based on results from a preliminary mouse 28-day dietary (Blanck, 2012 Study No. SA 07013) and mouse dose ranging finding study (Blanck, 2008 Study No. SA 07338, not submitted as part of this data package), where it was stated that a NOAEL was established at 1200 ppm for both sexes. However, the evaluator considers that in the preliminary mouse 28-day dietary study (Blanck, 2012 Study No. SA 07013) a NOAEL of 1200 ppm was established in females, and a NOAEL in males was determined to be 600 ppm.

Four groups of 10 C57BL/6J mice/sex/dose were administered BYI 02960 at 0, 100, 500, or 2500 ppm in feed daily for 92, 94, or 95 days, and mean doses were determined to be 0, 15.6, 80.6, and 407 mg/kg bw/day in males, and 0, 18.8, 98.1, and 473 mg/kg bw/day in females, respectively (Table KIIA 5.3.2/02-1).

Animals were assigned to dose groups by computerised, stratified randomisation, such that bodyweights were within 20% of the mean bodyweight on the day of randomisation. Animal housing and husbandry were in accordance with the provisions of the *Guide to the Care and Use of Laboratory Animals* (USPHS-NIH Publication No. 86-23) and "Le Guide du Journal Officiel des Communautes Europeennes L358, 18 Decembre 1986, n° 86/609/CEE du 24 Novembre 1986".

Table KIIA 5.3.2/02-1. Study Design and achieved dose levels

Group	No./sex/dose	Nominal dietary dose (ppm)	Achieved mean dose level (mg/kg bw/d)	
			M	F
1	10	0	0	0
2	10	100	15.6	18.8
3	10	500	80.6	98.1
4	10	2500	407	473

Data excerpted from page 91 of the study report.

3. Diet preparation and analysis

The required amount of the test material was incorporated into the diet by dry mixing to achieve the nominal dietary concentrations at each dosing level. Two preparations (F1 and F2) were used in the study. When not in use the diet formulations were stored at approximately -18°C.

The homogeneity of BYI 02960 in diet was verified on the first preparation at the lowest and highest concentrations to demonstrate adequate formulation procedures. Achieved homogeneity were in the range of 90–98% of the nominal concentration.

The stability of BYI 02960 in feed under frozen storage or room temperature conditions was not examined in this study, with the study authors instead citing stability analyses from Amir-Tahmasseb (2008; Study No. SA 07360), and from the 90-day dietary study in rats by Odin-Feurtet (2012; SA 07294). They concluded that the studies showed the test substance in feed at 50 to 2000 ppm to be stable over 79 days at ambient temperatures, and at 2500 ppm to be stable over 102 days at ambient temperatures.

4. Statistics

Statistical methods employed in this study are listed in Table KIIA 5.3.2/02-2.

Table KIIA 5.3.2/02-2. Statistics used in data analyses

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant (p>0.05)	**If preliminary test is significant (p≤0.05)**
Terminal body weight, body weight gain, clinical chemistry, organ weight (absolute and relative)	Bartlett Test	Analysis of variance followed by 2-sided Dunnett's test if significant	Kruskal-Wallis test followed by 2-sided Dunn's test if significant
Body weight and mean food consumption per day parameters	Bartlett Test	Analysis of variance followed by 2-sided Dunnett's test if significant	Bartlett Test on log transformed data: if significant then Kruskal-Wallis test followed by 2-sided Dunn's test if significant; Otherwise ANOVA followed by Dunnett tests on log transformed data if significant

C. METHODS

1. Observations

Animals were observed twice daily for mortality and morbidity (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily. Animals were also examined weekly for detailed clinical signs of toxicity, and the nature, onset, severity, duration and recovery of clinical signs recorded.

Cage and cage trays were inspected daily for evidence of ill health such as blood or loose faeces.

2. Body weights

All animals were weighed at study initiation and at necropsy, and on a weekly basis over the dosing period. Body weight change was calculated for each week and for the total study duration.

3. Food consumption, food efficiency and daily intake

   Food consumption was recorded for each animal weekly during the dosing period. Daily intake was calculated from food consumption and body weight data.

   Food use efficiency estimates were not undertaken.

4. Clinical pathology (clinical chemistry only)

   Blood samples were collected from all animals (anesthetized by inhalation of isoflurane) following an overnight fast on study day 93, 94, or 95 by retro-orbital venous plexus puncture. The following clinical chemistry parameters were evaluated.

Clinical Chemistry

aspartate aminotransferase	alkaline phosphatase
alanine aminotransferase	total protein
total bilirubin	albumin
creatinine	urea
total cholesterol	

5. Plasma concentration

   Blood (non-fasted) was collected from 5 males and 5 females of each dose group on study day 90, and immediately frozen at -20°C, for future determination of plasma concentration of test substance. Prior to blood collection, animals were anesthetized with isoflurane but were not diet fasted. Samples were evaluated by high performance liquid chromatography/tandem mass spectroscopy (HPLC-MS/MS).

6. Sacrifice and pathology

   At termination (study day 93, 94 or 95), animals were sacrificed by exsanguination under deep anesthesia (inhalation of isoflurane) following an overnight fast. Gross examinations were performed on all animals. Organs that were weighed are listed in Table KIIA 5.3.2/02-3. Relative organ weight to body weight and to brain weight ratios were calculated. For animals in the control and high dose groups (i.e. 2500 ppm) all the tissues (including gross lesions) indicated in Table KIIA 5.3.2/02-3 were collected and processed to slides and evaluated microscopically. For all other dose groups, only tissues from the liver, kidney, lung, thyroid gland tissue and gross lesions were collected, processed and evaluated microscopically.

Table KIIA 5.3.2/02-3: Organs/tissues collected for pathological examination

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted#
Adrenal gland	X	X	X
Aorta		X	X
Bone with bone marrow, sternum		X	X
Bone marrow smear^		X	
Brain	X	X	X
Epididymis		X	X
Esophagus		X	X
Eye (with optic nerve)		X	X
Harderian gland		X	X
Heart	X	X	X
Gallbladder		X	X
Kidney	X	X	X
Intestines (duodenum, jejunum, ileum, cecum, colon, rectum)		X	X
Lacrymal exorbital gland		X	
Larynx/pharynx		X	
Liver	X	X	X
Lung		X	X
Lymph nodes (submaxillary, mesenteric)		X	X
Mammary gland		X	X
Nasal cavities		X	
Ovary		X	X
Pancreas		X	X
Parathyroid gland		X	X
Pituitary		X	X
Prostate		X	X
Salivary gland, submaxillary		X	X
Sciatic nerve		X	X
Skeletal muscle		X	X
Seminal vesicle		X	X
Skin		X	X
Spinal cord, (cervical, lumbar, thoracic)		X	X
Spleen	X	X	X
Stomach		X	X
Testis	X	X	X
Thymus	X	X	X
Thyroid gland		X	X
Tongue		X	X
Trachea		X	X
Urinary bladder		X	X
Uterus with cervix	X	X	X
Vagina		X	X
Gross lesions		X	X

For animals in the low and mid dose groups (i.e. 100 and 500 ppm) in the main study, only tissues from the liver, kidney, lung, and thyroid along with gross lesions were processed to slides and examined microscopically. ^ A bone marrow smear of one femur was prepared for each animal, but were not examined for any group due to the absence of abnormal haematology findings.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

There were no observable test substance related clinical signs.

2. Mortality

Instances of unscheduled mortality were confined to a single female at 500 ppm (Animal No. ST3F1298) which was humanely sacrificed on study day 61. Necropsy revealed oesophageal dilatation and a tear, food content in the thoracic cavity, enlarged adrenal glands, dark liver, white foci on stomach, atrophic/small thymus and an enlarged bronchial lymph node. The study authors regarded the cause of death to be an initial oesophageal impaction followed by tearing and a locally extensive inflammatory reaction, and to be unrelated to the test substance. Given the absence of mortality at 2500 ppm and absence of lesions of the oesophagus in other dosing groups, this conclusion was considered by the evaluator to be justified.

B. BODY WEIGHT AND BODY WEIGHT GAIN

No body weight changes were observed at 100 ppm.

While a 10% decreased body weight gain compared to controls was observed in 500 ppm males across the duration of the study, final body weight was unaffected by treatment (i.e. within 1% of controls at study termination). This suggests that the decreased body weight gain in this case is not toxicologically adverse.

At 2500 ppm, a 3% mean bodyweight loss at 2500 ppm in males over study week 2 (compared to week 1 bodyweight) and females over study week 12 (compared to week 11 bodyweight; Table KIIA 5.3.2/02-4) was noted. Statistically significant differences in terminal bodyweights were also confined to 2500 ppm dosing in both sexes.

In males at 2500 ppm, bodyweights were statistically significantly ($p \leq 0.01$) lower relative to controls from study week 2 to 13, with the majority of the difference attributed to 36 and 70% lower bodyweight gains relative to controls over study weeks 2 and 3 (Table KIIA 5.3.2/02-5). Terminal bodyweights in 2500 ppm males were 10% lower than controls.

Terminal bodyweights in females at 2500 ppm were 5% ($p \leq 0.05$) lower than those of controls, with a 7% ($p \leq 0.01$) lower bodyweight in study week 1 and a consistent 3–8% decrease in body weight compared to controls during the dosing period. While a clear decreased body weight gain was observed in 2500 ppm females across the treatment period, the evaluator notes that the starting mean bodyweight was 4% lower at the start of the treatment period and 5% lower at study termination, which is also reflected in the 7% lower cumulative body weight gain value recorded at the final measurement point at day 92. Taking this into consideration, the evaluator considers it unlikely that there was a toxicologically adverse difference in terminal bodyweights for females at 2500 ppm in this case.

Table KIIA 5.3.2/02-4: Group mean bodyweights (g) for mice administered BYI 02960 for three months

Study day	Group mean bodyweight (grams±SD) and per cent change from control							
	M				F			
	Dose (ppm)							
	0	100	500	2500	0	100	500	2500
1	20±1	20.2±0.8 (1%)	20.3±0.6 (2%)	20.4±0.8 (2%)	16.8±0.7	16.6±0.6 (-1%)	16.7±0.6 (-1%)	16.2±1.4 (-4%)
8	21.4±1.1	21±0.9 (-2%)	20.9±0.8 (-2%)	**19.8±0.5 (-7%)****	18±0.5	17.6±0.7 (-2%)	17.4±0.8 (-3%)	**16.8±0.9 (-7%)****
15	22.4±1.2	22.3±1.1 (0%)	22±1.1 (-2%)	**20.4±0.6 (-9%)****	18.9±0.9	18.8±0.7 (-1%)	18.5±0.5 (-2%)	**17.9±1 (-5%)***
22	23.1±1.3	23.1±1 (0%)	22.5±1.3 (-3%)	**20.6±0.6 (-11%)****	19.8±0.6	19.4±0.9 (-2%)	19.2±0.5 (-3%)	**18.3±0.9 (-8%)****
29	23.5±1.2	23.5±0.9 (0%)	23±1 (-2%)	**21±0.6 (-11%)****	20.2±0.8	19.8±1.1 (-2%)	19.6±0.8 (-3%)	**18.7±0.7 (-7%)****
36	24.3±1.1	24.4±1 (0%)	23.8±1.2 (-2%)	**21.7±0.8 (-11%)****	20.6±1.1	20.6±0.9 (0%)	20.2±0.6 (-2%)	**19.5±0.6 (-5%)***
43	24.9±1.2	24.9±1.1 (0%)	24.5±1.2 (-2%)	**22.3±0.7 (-10%)****	20.7±1	21.1±0.8 (2%)	20.4±0.7 (-1%)	**20±0.7 (-3%)****
50	25.1±1.2	25±1 (0%)	24.6±1.1 (-2%)	**22.4±0.8 (-11%)****	21±0.7	20.8±1.1 (-1%)	20.7±0.6 (-1%)	**20±0.7 (-5%)***
57	25.7±1.1	25.8±1 (0%)	25.2±1.4 (-2%)	**23.2±0.8 (-10%)****	21.4±0.6	21.4±1 (0%)	20.8±1.6 (-3%)	**20.1±0.6 (-6%)****
64	26±1.2	26±1.3 (0%)	25.4±1.2 (-2%)	**23.3±1.1 (-10%)****	21.6±0.7	21.4±1.1 (-1%)	21.1±0.5 (-2%)	**20.1±0.5 (-7%)****
71	26.2±1.1	26.1±1.3 (0%)	25.4±1.1 (-3%)	**23.6±1 (-10%)****	21.5±0.7	21.1±1.2 (-2%)	21±0.6 (-2%)	**20±0.8 (-7%)****
78	26.7±1.2	26.8±1.1 (0%)	26.2±1 (-2%)	**23.9±0.8 (-10%)****	22±0.9	21.7±1 (-1%)	21.6±0.6 (-2%)	**20.8±0.7 (-5%)****
85	26.7±1.3	26.8±0.8 (0%)	26±1.2 (-3%)	**23.8±1 (-11%)****	22±0.9	21.6±1.2 (-2%)	21.3±0.9 (-3%)	**20.2±0.7 (-8%)****
92	27.2±1.3	27.1±0.8 (0%)	26.8±1.2 (-1%)	**24.4±0.8 (-10%)****	22.2±0.9	22.2±1.1 (0%)	21.8±0.8 (-2%)	**21.2±0.6 (-5%)***

* Significantly different from the control at $p \leq 0.05$ or **$p \leq 0.01$. Data excerpted from pages 71 to 74 of the study report, except for per cent change from controls, which was calculated by the evaluator using Microsoft Excel.

Table KIIA 5.3.2/02-5: Mean Cumulative body weight gain (g) for mice administered BYI 02960 for three months

	M				F			
	Dose (ppm)							
Study day	0	100	500	2500	0	100	500	2500
8	1.4±0.5	**0.8±0.5 (-43%)***	**0.6±0.4 (-57%)****	**-0.6±0.5 (-143%)****	1.1±0.4	1±0.3 (-9%)	0.7±0.6 (-36%)	**0.5±1.1 (-55%)****
15	2.4±0.5	2±0.7 (-17%)	1.7±0.9 (-29%)	**0±0.4 (-100%)****	2.1±0.6	2.2±0.5 (±5%)	1.8±0.5 (-14%)	1.7±1.3 (-19%)
22	3.1±0.6	2.9±0.9 (-6%)	**2.2±1.1 (-29%)***	**0.3±0.5 (-90%)****	2.9±0.8	2.9±0.7 (0%)	2.5±0.5 (-14%)	**2.0±1.6 (-31%)***
29	3.4±0.7	3.3±0.9 (-3%)	2.7±0.9 (-21%)	**0.6±0.5 (-82%)****	3.4±0.8	3.3±0.8 (-3%)	2.9±0.9 (-15%)	2.5±1.4 (-26%)
36	4.3±0.6	4.2±0.8 (-2%)	3.5±1 (-19%)	**1.3±0.8 (-70%)****	3.8±0.9	4.1±0.7 (8%)	3.4±0.8 (-11%)	3.3±1.4 (-13%)
43	4.8±0.7	4.7±1 (-2%)	4.2±1 (-13%)	**1.9±0.9 (-60%)****	3.9±0.9	4.6±0.6 (18%)	3.7±0.8 (-5%)	3.8±1.2 (-3%)
50	5±0.8	4.8±0.9 (-4%)	4.2±0.9 (-16%)	**2.1±0.7 (-58%)****	4.2±0.7	4.3±0.7 (2%)	4±0.7 (-5%)	3.8±1.4 (-10%)
57	5.7±0.8	5.5±1.5 (-4%)	4.9±1.2 (-14%)	**2.8±0.8 (-51%)****	4.6±0.7	4.8±0.7 (4%)	4.1±1.8 (-11%)	3.9±1.3 (-15%)
64	6±0.8	5.8±1.3 (-3%)	5.1±1.1 (-15%)	**3±0.8 (-50%)****	4.7±0.7	4.8±0.9 (2%)	4.4±0.7 (-6%)	3.9±1.1 (-17%)
71	6.2±0.9	5.9±1.3 (-5%)	**5.1±1 (-18%)***	**3.2±0.7 (-48%)****	4.7±0.9	4.5±0.9 (-4%)	4.3±0.7 (-9%)	3.8±1.2 (-19%)
78	6.6±0.9	6.6±1.2 (0%)	5.9±0.9 (-11%)	**3.6±0.6 (-45%)****	5.2±0.9	5.2±0.7 (0%)	5±0.8 (-4%)	4.5±1.2 (-13%)
85	6.7±1	6.5±1 (-3%)	5.7±1.1 (-15%)	**3.5±0.8 (-48%)****	5.2±0.9	5±1 (-4%)	4.6±1.1 (-12%)	4±1.4 (-23%)
92	7.2±1.2	6.9±0.8 (-4%)	6.5±1 (-10%)	**4.1±0.8 (-43%)****	5.4±0.7	5.7±0.9 (6%)	5.2±0.9 (-4%)	5±1.2 (-7%)

'*' statistically significant at the p≤0.05 or ** p≤0.01 level. Data excerpted from pages 81 to 84 of the study report, and per cent changes (rounded to an integer) from control were calculated manually by the evaluator using Microsoft Excel.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

In a similar pattern to body weight observations, decreased food consumption was observed in animals administered 2500 ppm test substance, particularly in study week 1–2, where up to 11% lower food consumption was observed in both sexes when compared to controls (Table KIIA 5.3.2/02-6). Mean food consumption in females remained 5 to 10% lower than controls over the course of the study, while in males only a 3–5% decrease in food consumption compared with controls was observed.

Estimates of food consumption in g food/kg bw/d and food use efficiency were not provided, and were not calculated by the evaluator.

The study authors regarded the food consumption findings at 2500 ppm to be unrelated to the test substance for males, but to be test substance related for females. The evaluator notes that while food consumption may not have been affected in this case for 2500 ppm male animals, the clear decreased body weight gain observed suggests a decrease in food use efficiency which should be considered

treatment-related in the context of this study. Overall, food consumption patterns in 2500 ppm animals are considered to be affected by treatment with the test substance.

Table KIIA 5.3.2/02-6: Food consumption

Study day	Group mean food consumption (grams/day+SD) and per cent change from control							
	M				F			
	Dose (ppm)							
	0	100	500	2500	0	100	500	2500
8	3.8±0.4	3.9±0.2 **(+3%)**	3.8±0.2 **(0%)**	3.6±0.4 **(-5%)**	3.6±0.3	3.6±0.3 **(0%)**	3.5±0.3 **(-3%)**	3.2±0.5 **(-11%)***
15	3.8±0.2	3.8±0.2 **(0%)**	3.7±0.2 **(-3%)**	3.4±0.3 **(-11%)****	3.8±0.3	3.6±0.2 **(-5%)**	3.6±0.2 **(-5%)**	3.5±0.6 **(-8%)**
22	3.8±0.2	3.9±0.3 **(+3%)**	3.9±0.2 **(+3%)**	3.6±0.3 **(-5%)**	3.9±0.3	3.7±0.2 **(-5%)**	3.9±0.3 **(0%)**	3.5±0.4 **(-10%)***
29	3.7±0.2	3.8±0.3 **(+3%)**	4±0.1 **(+8%)***	3.6±0.1 **(-3%)**	3.9±0.3	3.7±0.3 **(-5%)**	3.9±0.4 **(0%)**	3.6±0.4 **(-8%)**
36	3.7±0.1	3.8±0.3 **(+3%)**	3.9±0.2 **(+5%)**	3.5±0.3 **(-5%)**	4±0.4	3.8±0.4 **(-5%)**	3.9±0.5 **(-3%)**	3.6±0.4 **(-10%)**
43	3.7±0.2	3.8±0.3 **(+3%)**	4±0.3 **(+8%)***	3.7±0.3 **(0%)**	4±0.5	4±0.6 **(0%)**	4±0.5 **(0%)**	3.8±0.5 **(-5%)**
50	3.7±0.2	3.8±0.3 **(+3%)**	3.9±0.3 **(+5%)**	3.8±0.2 **(+3%)**	4.1±0.5	4±0.4 **(-2%)**	4.1±0.6 **(0%)**	3.7±0.5 **(-10%)**
57	3.7±0.2	3.9±0.8 **(+5%)**	4±0.4 **(+8%)**	3.6±0.2 **(-3%)**	4.1±0.6	4±0.3 **(-2%)**	4±0.7 **(-2%)**	3.8±0.3 **(-7%)**
64	3.7±0.2	3.8±0.5 **(+3%)**	4±0.4 **(+8%)***	3.7±0.2 **(0%)**	4.2±0.8	4±0.3 **(-5%)**	4.2±0.5 **(0%)**	3.9±0.4 **(-7%)**
71	3.7±0.2	3.8±0.2 **(+3%)**	3.8±0.2 **(+3%)**	3.6±0.2 **(-3%)**	4±0.6	3.8±0.4 **(-5%)**	4±0.6 **(0%)**	3.7±0.3 **(-8%)**
78	3.8±0.4	3.9±0.2 **(+3%)**	3.9±0.2 **(+3%)**	3.7±0.2 **(-3%)**	4.2±0.6	4±0.3 **(-5%)**	4.2±0.5 **(0%)**	4±0.4 **(-5%)**
85	3.7±0.2	3.8±0.2 **(+3%)**	3.9±0.2 **(+5%)***	3.6±0.2 **(-3%)**	4±0.6	3.9±0.2 **(-3%)**	4.1±0.6 **(+2%)**	3.7±0.4 **(-8%)**
92	3.9±0.3	4±0.2 **(+3%)**	4±0.2 **(+3%)**	3.7±0.1 **(-5%)**	4.1±0.8	4±0.3 **(-2%)**	4.3±0.5 **(+5%)**	4±0.3 **(-2%)**

* Significantly different from the control at p ≤0.05 or **p ≤0.01. Data excerpted from pages 86 to 89 of the study report, except for per cent change from controls, which was calculated by the evaluator using Microsoft Excel.

D. CLINICAL PATHOLOGY (clinical chemistry)

Statistically significant changes in clinical chemistry parameters were confined to 2500 ppm in both sexes (Table KIIA 5.3.2/02-7). Dose dependent trends for

decreased serum cholesterol was seen in both sexes, reaching statistical significance at 2500 ppm at 30/24% (M/F) decreases relative to controls ($p \leq 0.01$). An associated finding was a statistically significant 8% decreased ($p \leq 0.01$) serum albumin concentration in females at 2500 ppm and 5% decreased serum total protein in both sexes ($p \leq 0.05$ in males; $p \leq 0.01$ in females).

Increased serum urea levels at all doses in males and from 500 ppm in females, though statistical significance was only observed at 2500 ppm with increases of 51/19% in M/F ($p \leq 0.01$ in males; $p \leq 0.05$ in females), suggesting that treatment-related change was observed at 2500 ppm only.

A range of dose dependent changes in parameters most likely representing hepatic dysfunction and hepatotoxicity were seen at 2500 ppm, but with differences in magnitude between the sexes. In males at 2500 ppm, serum ALP activity was increased by 38% ($p \leq 0.01$) relative to controls, whereas only a slight non-statistically significant 10% increase was seen in females. Alanine aminotransferase activity was increased by 106% in females ($p \leq 0.05$) relative to controls, and only by 30% in males (not statistically significant), while aspartate aminotransferase activity was increased by 19/36% (M/F) relative to controls without statistical significance.

Table KIIA 5.3.2/02-7: Clinical chemistry from mice administered BYI 02960 for three months

	Group mean value±SD and per cent change from control							
	M				F			
	Dose (ppm)							
Parameter	**0**	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
Total cholesterol (mmol/l)	1.91±0.18 -	1.87±0.10 **(-2%)**	1.77±0.20 **(-7%)**	1.34±0.14 **(-30%)****	1.52±0.16 -	1.50±0.20 **(-1%)**	1.42±0.11 **(-7%)**	1.16±0.17 **(-24%)****
Alkaline phosphatase (ALP) (IU/l)	76±11 -	74±8 **(-3%)**	81±10 **(+7%)**	105±11 **(+38%)****	131±14 -	129±7 **(-2%)**	134±15 **(+2%)**	144±15 **(+10%)**
Alanine aminotransferase (ALT) (IU/l)	27±5 -	32±14 **(+19%)**	32±18 **(+19%)**	35±9 **(+30%)**	36±10 -	38±10 **(+6%)**	38±9 **(+6%)**	74±38 **(+106%)***
Aspartate aminotransferase (AST) (IU/l)	88±15 -	91±26 **(+3%)**	95±33 **(+8%)**	105±20 **(+19%)**	130±21 -	129±41 **(-1%)**	132±36 **(+2%)**	177±68 **(+36%)**
Urea (mmol/l)	11.14±1.23 -	14.04±3.17 **(+26%)**	13.98±3.15 **(+25%)**	16.78±2.85 **(+51%)****	12.76±1.22 -	13.31±1.51 **(+4%)**	14.79±2.13 **(+16%)**	15.23±2.46 **(+19%)***
Total protein (g/l)	59±2 -	57±2 **(-3%)**	59±2 **(0%)**	56±4 **(-5%)***	58±2 -	58±2 **(0%)**	57±2 **(-2%)**	55±2 **(-5%)****
Albumin (g/l)	34±0 -	34±1 **(0%)**	35±2 **(-3%)**	33±2 **(-3%)**	36±1 -	35±1 **(-3%)**	35±1 **(-3%)**	33±1 **(-8%)****

* Significantly different from the control at $p \leq 0.05$ or **$p \leq 0.01$. Data excerpted from page 56 of the study report, except for values for 100 and 500 ppm groups, which were extracted pages 93 and 94.

F. PLASMA CONCENTRATION OF TEST SUBSTANCE

Test substance concentrations in blood are reported in Table KIIA 5.3.2/02-8 below. In contrast to the 90-day dietary study in rats (Odin-Feurtet, 2012: Report SA 07294), test substance concentrations in plasma at 90 days were higher in males than in females at all concentrations of test substance in feed. At 500 ppm relative to 100 ppm (5-fold increase), both sexes experienced an above-linear increase in plasma concentrations vs administered concentration of test substance (i.e., approx. 5.6-fold in M and 7.0-fold in F), while at 2500 ppm relative to 100 ppm (25-fold increase) males experienced a sub-linear increase in plasma concentration vs administered concentration of test substance of 22-fold while females experienced an above linear increase of 27.6-fold. Overall, a generally linear correlation between administered concentrations of BYI 02960 in the diet and concentrations in plasma was observed.

Table KIIA 5.3.2/02-8. Individual & group mean plasma concentrations of BYI 02960 (mg/L±SD)

N=5	Dose (ppm)			
	0	**100**	**500**	**2500**
Males				
Animal Nº's [(B)]	**#1240-1244**	**1260 to 1264**	**1280 to 1284**	**1300 to 1304**
	<LLOQ	2.03	13.4	30.7
	<LLOQ	2.68	11.2	48.5
	<LLOQ	2.03	8.30	42.5
	<LLOQ	1.44	11.5	48.2
	<LLOQ	1.53	9.66	44.2
Group Mean	<LLOQ	1.94±0.50	10.8±1.93	42.8±7.25
Fold-increase from 100 ppm	**-**	***1***	**5.6**	**22.1**
Females				
Animal Nº's	**#1250 to 1254**	**1270 to 1274**	**1290 to 1294**	**1310 to 1314**
	<LLOQ	0.968	10.4	43.7
	<LLOQ	2.74	8.03	30.9
	<LLOQ	1.10	9.22	35.3
	<LLOQ	0.739	9.05	33.4
	<LLOQ	0.623	6.92	26.7
Group Mean	<LLOQ	1.23±0.86	8.72±1.31	34.0±6.31
Fold-increase from 100 ppm	**-**	**1**	**7.1**	**27.6**
Fold-change from males	**-**	***0.63 (-37%)***	***0.81 (-19%)***	***0.79 (-21%)***

Lower limit of quantification (LLOQ) = 0.025 mg/L. 'B' – the first four characters in each animal number have been omitted in the above table but are presented in full elsewhere in this study evaluation. Data excerpted from pages 15 and 16 of the study report, except for fold-increases from 100 ppm values and fold increase from males, which were calculated by the evaluator using Microsoft Excel.

G. SACRIFICE AND PATHOLOGY

1. Organ weights

Statistically significant changes in absolute or relative (to brain or bodyweights) weights were confined to 2500 ppm in both sexes, with a 12-13% ($p<0.01$) increase in absolute and relative (to brain weight) liver weight

in females, and an 11% ($p<0.01$) decrease in absolute and relative (to brain weight) kidney weight in males (Table KIIA 5.3.2/02-9). A 20/18% (M/F, $p\leq0.01$) increase in relative (to bodyweight) liver weights in both sexes at 2500 ppm was also observed. Increased relative (to body weight) brain weights were noted in 2500 ppm males (+12%; $p<0.01$), which were considered to be secondary to the reduced bodyweights noted in high dose males.

All other organ weight changes were considered incidental to treatment.

Table KIIA 5.3.2/02-9. Organ weight findings

Organ parameter	M				F			
	Dose (ppm)							
	0	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
Terminal body weight (g)	23.0±1.2	23.0±0.9 (0%)	22.5±1.1 (-2%)	20.4±0.7 (-11%)**	18.3±0.8	18.1±1.0 (-1%)	18.2±0.5 (-1%)	17.6±0.6 (-4%)
Absolute liver weight (g)	0.96±0.06	1.02±0.08 (+6%)	0.95±0.07 (-1%)	1.02±0.06 (+6%)	0.82±0.04	0.82±0.06 (0%)	0.84±0.08 (+2%)	0.92±0.07 **(+12%)****
Liver to body weight ratio (%)	4.17±0.212	4.422±0.264 (+6%)	4.207±0.149 (+1%)	4.989±0.362 **(+20%)****	4.474±0.209	4.498±0.186 (+1%)	4.609±0.433 (+3%)	5.264±0.437 **(+18%)****
Liver to brain weight ratio (%)	214.9±12.390	223.2±18.445 (+4%)	215.2±14.193 (0%)	229.9±17.249 (+7%)	181.5±10.59	179.3±11.537 (-1%)	191.0±18.70 (+5%)	205.62±16.40 **(+13%)****
Absolute kidney weight (g)	0.35±0.03	0.35±0.03 (0%)	0.32±0.03 (-9%)	0.31±0.02 **(-11%)****	0.26±0.01	0.27±0.02 (+4%)	0.26±0.01 (0%)	0.25±0.01 (-4%)
Kidney to body weight ratio (%)	1.514±0.115	1.531±0.098 (+1%)	1.437±0.097 (-5%)	1.515±0.092 (0%)	1.439±0.060	1.474±0.127 (+2%)	1.417±0.050 (-2%)	1.425±0.045 (-1%)
Kidney to brain weight ratio (%)	78.01±6.005	77.26±6.434 (-1%)	73.41±5.083 (-6%)	69.79±4.180 **(-11%)****	58.32±2.117	58.67±4.816 (+1%)	58.72±3.072 (+1%)	55.71±2.477 (-4%)

* Significantly different from the control at $p \leq 0.05$ or **$p\leq0.01$. Data excerpted from pages 57 and 96-101 of the study report.

2. Gross pathology and histopathology

Grossly, an increased incidence of pale liver was noted in females at 2500 ppm (6/10 versus 0 in controls).

Changes in necropsy and histopathology were confined to the 2500 ppm groups (Table KIIA 5.3.2/02-10). The severity of diffuse centrilobular hepatocellular hypertrophy appeared to be increased at 2500 ppm in both sexes, but interpreting this finding was complicated by all animals in all groups having diffuse centrilobular hepatocellular hypertrophy of at least minimal severity. However, the only groups to report moderate severity for this effect were the 2500 ppm group of both sexes (4/10 males and 5/10 females). This finding is typical of adaptive responses to xenobiotic detoxification (*i.e.*, is adaptive), but together with changes at 2500 ppm comprising 30/24% (M/F, $p<0.01$) decreases in serum cholesterol, 38% ($p<0.01$) increase in alkaline phosphatase activity in males, and the 106% increase in alanine aminotransferase activity in females, it is considered sufficient evidence for adverse hepatocellular toxicity present in both sexes at this dose.

Additionally, a loss in extent of normal multifocal/diffuse cortical epithelial cell vacuolation in the kidney was seen in males at 2500 ppmThe changes at 2500 ppm in males were marked, with a complete absence of vacuolation, and may be linked to statistically significant increase ($p\leq0.05$) of 51% in serum urea and a 5% decrease ($p\leq0.05$) in serum protein, and an 11% ($p<0.01$) decrease in absolute and relative (to brain weight) kidney weight in males at this dose. Therefore the absence of multifocal/diffuse cortical epithelial cell vacuolation at this dose can in this case be considered to be one of several symptoms indicating test substance related, toxicologically adverse renal toxicity in males at 2500 ppm.

Table KIIA 5.3.2/02-10: Necropsy and histological findings at scheduled sacrifice

	Dose (ppm)							
	Males				**Females**			
	0	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
Diffuse centrilobular hepatocellular hypertrophy								
Minimal	6	3	8	0	7	7	6	2
Slight	4	7	2	6	3	3	3	3
Moderate	0	0	0	4	0	0	0	5
Total	**10/10**	**10/10**	**10/10**	**10/10**	**10/10**	**10/10**	**9/9**	**10/10**
Multifocal/diffuse corticoepithelial vacuolation (kidney)								
Minimal	7	4	5	0	0	0	0	0
Slight	3	2	3	0	0	0	0	0
Moderate	0	2	0	0	0	0	0	0
Total	**10/10**	**8/10**	**8/10**	**0/10**	**0/10**	**0/10**	**0/9**	**0/10**

N=10 for all groups but females at 500 ppm, due to the humane sacrifice of one animal in this group on study day 61. Statistical analysis was not undertaken. Data excerpted from pages 58 of the study report.

III. CONCLUSION

Under the conditions of this study BYI02960 did not affect mortality nor produce clinical signs in treated animals. No treatment-related findings were observed at 100 ppm or 500 ppm, noting the decreased body weight gain at 500 ppm was not considered a toxicologically adverse effect.

At 2500 ppm, statistically significant decreases in bodyweight gains in males compared with controls were noted over the treatment period. Clinical chemistry analyses reported treatment-related decreases of 30/24% (M/F) in serum cholesterol levels, 51/19% (M/F) increases in plasma urea concentrations, 5% (both sexes) decreases in protein concentrations in plasma, 38% increases in plasma ALP activity in males, and 106% increases in alanine aminotransferase activity and 36% increase in aspartate aminotransferase activity in females. In terminal findings, an increased absolute (+12%) and relative (to bodyweight, +18%; to brain weight, +13%) liver weights in females, and decreased absolute and relative kidney weights (to brain weight; -11%) in males was observed. Pale liver was observed in 6/10 2500 ppm females, and histopathological examination reported slightly increased severity of hepatocellular vacuolation in both sexes, along with the loss of normal multifocal/diffuse cortical epithelial vacuolation in kidney of males.

The increased severity of diffuse centrilobular hepatocellular hypertrophy and the reported liver-related organ weight and clinical chemistry changes are considered to be consistent with toxicologically adverse hepatocellular toxicity in both sexes at 2500 ppm.

Additionally, in males, the loss of multifocal/diffuse cortical epithelial cell vacuolation, combined with the changes in kidney-related clinical chemistry parameters and decreases in absolute and relative (to brain weight) kidney weight, collectively suggest test substance related, toxicologically adverse renal toxicity in males at 2500 ppm.

The NOAEL was 500 ppm (81 mg/kg/day) for both sexes. The LOAEL was 2500 ppm (407 mg/kg/day) based on based on consistent decreases in body weight (>10%) with associated reduction in body weight gains in male, liver effects (↑ ALP and ALT, ↓ in total cholesterol, ↑ in absolute and relative liver weights, increased incidence of moderate diffuse hepatocellular hypertrophy). Kidney effects (changes in clinical chemistry parameters and loss of multifocal/diffuse cortical epithelial cell vacuolation & increases in urea),.

This study is classified as fully reliable (acceptable/guideline) and does not satisfy guideline requirements for a subchronic oral toxicity in mice [OPPTS 870.3100; OECD 408 (1998)]. Although hematological parameters were not assessed in this study, they were assessed in the 18-month oncogenicity study in mice.

Primary reviewer: Australia
Secondary review: US EPA & PMRA

Report: Kennel P (2012) BCS-AA56716 (Difluoroacetic acid) - 90-day toxicity study in the rat by dietary administration. Bayer S.A.S., Bayer, CropScience, Sophia Antipolis, France. Study No. SA 10324. February. Sponsor study ID: Lynx-PSI N^{o} TXRVP092. February. MRID 48844113. Unpublished.

Sponsor: Bayer CropScience AG, Alfred Nobel Str. 50, 40789 Monheim, Germany

Guidelines: OPPTS 870.3100 (1998)
EEC Directiove 2001/59/EC, method B.26 (August)
OECD 408 (1998)
MAFF Nousan 8147 (2000)

Deviations: The stability of the test substance in prepared diet was not examined over the whole duration of the study. This was considered not to have affected the overall reliability of thes study findings.

GLP: Yes Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a 90-day dietary study (MRID 48844113), BCS-AA56716 (97.1%; batch BCOO5984-5-8) was administered to Wistar [Rj:WI (IOPS HAN)] rats (10/sex/dose) at concentrations of 0, 200, 1000, or 6000 ppm (0/0, 12.7/15.6, 66.2/78.7, or 380/472 mg/kg bw/d, respectively). BCS-AA56716 (difluoroacetic acid) is a plant and soil metabolite of BYI02960.

Parameters evaluated included bodyweight, bodyweight gain, food consumption, clinical signs, Functional Observational Battery parameters, exploratory locomotor activity, haematology, clinical chemistry, urinalysis, ophthalmology, organ weights, gross and microscopic pathology.

Under the conditions of this study BCS-AA56716 did not produced test substance-related effects on survival, clinical signs of toxicity, functional observational battery observations, ophthalmological observations, or organ weights.

In males at 1000 ppm, mean bodyweight by study day 8 was 4% lower than controls, 9% ($p \leq 0.01$) lower than controls by study day 29, and terminal bodyweights were 12% ($p \leq 0.01$) lower than controls at study day 92. Lower mean bodyweight and decreased bodyweight gain were also noted at 6000 ppm, with a mean bodyweight 4% lower than controls by study day 29, 6% lower by study day 57, with no further decreases through to study termination (all values were not statistically significant).

In females at 1000 ppm, mean bodyweight was 5% lower than controls at study day 57, and were 4% lower than controls by study day 92 (no statistical significance observed). Lower mean bodyweights during the treatment period were also observed at 6000 ppm in females, with a mean bodyweight 6% ($p \leq 0.05$) lower than controls at study day 29, 7% ($p \leq 0.05$) lower at study day 57 ($p \leq 0.05$), and 9% ($p \leq 0.01$) lower at study day 92.

Decreased bodyweight gains in both sexes at 1000 and 6000 ppm was accompanied by corresponding decreases in absolute (g/d) food consumption from study day 4 onwards (earliest time point examined).

In haematology observations, a 9% lower haemoglobin, 6% lower mean corpuscular volume, and 8% lower mean corpuscular haemoglobin were noted in 1000 ppm females relative to controls,. These findings all displayed poor dose dependency, with similar decreasese reported at 6000 ppm. .

In clinical chemistry observations, Serum glucose concentrations and serum bilirubin were decreased across all dose levels compared with controls; however, noting the direction of change for the reported effects (decreases, rather than increases), the effects were not considered toxicologically adverse.

A range of urinalysis parameters at ≥ 1000 ppm (including urinary volume, ketone levels and urinary pH) were considered to be treatment-related, but unlikely to be toxicologically significant given the lack of histopathology and clinical pathology changes.

Test substance related findings at gross necropsy were confined to black foci in the stomach gland of both sexes at 1000 ppm (10/20% incidence for M/F) and 6000 ppm (30%/20% incidence for M/F). The finding was associated at the histopathological level with focal glandular erosion/necrosis in the stomach, and considered to be toxicologically adverse.

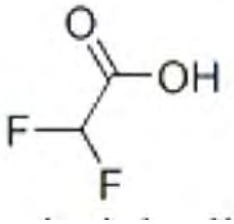
The No Observed Adverse Effect Level (NOAEL) in this study is 200 ppm for both sexes (12.7/15.6 mg/kg bw/d for M/F). The LOAEL was 1000 ppm (66.2/78.7 mg/kg bw/d for M/F), based on decreased bodyweight gains in males, resulting in 12% lower terminal bodyweights relative to controls ($p \leq 0.01$), associated decreases in food consumption, black foci in the glandular part of the stomach (10/20% incidence for M/F) for both sexes that correlated histopathologically with focal glandular erosion/necrosis, and (in females only) statistically significant ($p \leq 0.01$) decreases in plasma haemoglobin (-9%), mean corpuscular volume (-6%), mean corpuscular haemoglobin (-8%) and haematocrit (-7%).

This study is classified as fully reliable (acceptable/guideline) and satisfies guideline requirements for a subchronic oral toxicity in rats [OPPTS 870.3100; OECD 408 (1998)].

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BCS-AA56716
(difluoroacetic acid)



Lot/Batch #:	BCOO5984-5-8
Purity:	97.1% (as analysed at 6 Dec 2010)
Description:	Colourless liquid
CAS #	381-73-7
Stability of test compound:	Stored in an air-tight, light resistant container at room temperature. Stated to be stable for six months at 20±10ºC.

2. Vehicle and/or positive control: Untreated diet. No positive control used in this study

3. Test animals

Species:	Rat
Strain:	Wistar Rj:WI (IOPS HAN)
Age at dosing:	Approx. 6 to 7 weeks
Weight at dosing:	183 to 208 g (males); 150 to 177 g (females)
Source:	R. Janvier, Le Genest St Isle, France
Acclimation period:	6 days
Diet:	A04CP1-10 from S.A.F.E. ((Scientific Animal Food and Engineering, Augy, France) *ad libitum* except for an overnight fast prior to urinalysis on study days 87 or 88. During the test period, test substance was incorporated into the feed of all animals except negative controls.
Water:	Filtered and softened tap water, *ad libitum*, except for an overnight fast prior to urinalysis on study days 87 or 88.
Housing:	By groups of 5 per sex in suspended, stainless steel and wire mesh cages.

4. Environmental conditions

Temperature:	22 to 24°C
Humidity:	40 to 70%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion
13-Jan-2011 to 03-Aug-2011

2. Animal assignment and treatment

Four groups of 10 rats/sex/dose received BCS-AA56716 in feed at concentrations of 0, 200, 1000, or 6000 ppm daily for 93 to 95 days (Table KIIA 5.8/06-1).

Animals were assigned to dose groups by computerised randomisation such that mean group bodyweights were within ±20% of group mean bodyweight on the day of randomisation. A negative control group received untreated diet.

Table KIIA 5.8/06-1: Study Design and Compound Intakes

Conc. in diet (ppm)[a]	Mean daily compound intakes (mg/kg bw/d) ±SD [N=10]		Animal No.'s	
	M	F	M	F
0	0	0	T1M0002 to 0011	T1F0012 to 0021
200	12.7±3.4	15.6±2.4	T2M0022 to 0031	T2F0032 to 0041
1000	66.2±14.2	78.7±9.7	T3M0042 to 0051	T3F0052 to 0061
6000	380±95.0	472±57.7	T4M0062 to 0071	T4F0072 to 0081

Unadjusted for compound purity or degradation. Mean intakes provided by the study authors, and SD calculated by the evaluator using weekly means only and the Standard deviation function in Microsoft Excel (*i.e.*, Standard deviations are an underestimate as they exclude standard deviations within each week). Data excerpted from pages 46 and 87 of the study report, except for standard deviations, which were calculated by the evaluator (see explanation below).

The dose levels selected for this current study were based on results from a 14-day dietary toxicity study in rats with BCS-AA56716 provided in the applicant's submission (Kennel, 2012; Study No. SA 10323). In the study, a 13/12% (M/F) decrease in overall bodyweight gain relative to controls was seen in the highest dose tested of 8000 ppm, resulting in a 6/7% (M/F) lower terminal bodyweight relative to controls. Additionally, a 54/45% ($p \leq 0.01$) lower serum glucose and 27% higher concentration of serum urea relative to controls was noted at 8000 ppm in males, with similar effects observed in females at 2000 ppm. The study authors set a NOAEL of 2000 ppm (~187 mg/kg bw/d) in males and 500 ppm (~51 mg/kg bw/d) in females, based on adverse effects at 8000 ppm and 2000 ppm, respectively.

3. Diet preparation and analysis

The test substance was incorporated into the diet by dry mixing to achieve concentrations of 200, 1000 or 6000 ppm. Two preparations were made for the study, and stored at -18°C when not in use for a maximum of 49 days, followed by a maximum storage of eight days at room temperature.

Samples collected on 17 Jan 2011 from the first prepared batches at 200 and 6000 ppm were subjected to analysis of homogeneity/achieved concentration by LC-MS/MS. Samples collected on 7 March 2011 from the second prepared batches at these concentrations as well as the 1000 ppm batches were subjected to analysis of achieved concentrations only. Collected samples were stored at -20°C immediately after collection. Collected samples were then thawed and left for one day (17 Jan samples) or three days (7 March samples) at ambient temperatures, and then analysed. Additionally, on both sample collection dates at least one sample of control diet (0 ppm nominal) was collected and used for preparation of standards.

Homogeneity analyses reported acceptable variations within samples (+3.3% and -5.5% variation between sampling layers from 200 and 6000 ppm diets), while concentration analyses reported variations from nominal concentrations of +0.6–9.7% (200 ppm), -0.8–+2.6% (1000 ppm) and -8.8–+2.3% (6000 ppm).

Stability analysis was not undertaken. Instead, the study authors cited results of a study by

Andre (2011) of the stability BCS-AA56716 in feed, however, the study was not provided in the applicant's submission. According to the analytical report, Andre (2011) found that concentrations after 11 days at room temperature were 101.75% and 90.5% of nominal at 200 and 8000 ppm, respectively. After 32 days storage at approx. -18°C and a further 10 days at room temperature, concentrations were 114.25% and 95.10% of nominal concentrations at 200 and 8000 ppm, respectively. Noting that similar rates of degradation were observed between frozen and ambient samples, the study authors did not expect further degradation would result from extending storage at -18°C for 17 days (49 days in total), though noted that the use of diet frozen for an additional 17 days was a protocol deviation. The evaluator concurs that stability is unlikely to deviate meaningfully with additional frozen storage time in this case.

Based on this information, it can be concluded that the animals received the targeted dietary concentrations of test substance during the study, and no concentration adjustment factor is required.

4. Statistics

Statistical methods employed in this study are listed in Table KIIA 5.8/06-2, and were carried out using Pristima System, version 6.1.0, Build 19.

Table KIIA 5.8/06-2. Statistics used in data analyses.

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant (p>0.05)	**If preliminary test is significant (p≤0.05)**
Body weight change Haematology (except for rbc, plt, wbc, neutrophil, lymphocytes, and reticulocyte counts) Clinical chemistry Quantitative urinalysis (except pH) Organ weights (absolute & relative) FOB parameters Spontaneous motor activity	**Bartlett Test**	**Analysis of variance**, followed by 2-sided **Dunnett's test if significant**	**Kruskal-Wallis test** followed by 2-sided **Dunn test if significant**
Terminal bodyweight Haematology (rbc, plt, wbc, neutrophil, lymphocytes, and reticulocyte counts)	**Bartlett Test**	Analysis of variance followed by 2-sided **Dunnett's test if significant**	**Bartlett Test** on log-transformed data**;** if significant then **Kruskal-Wallis test** followed by 2-sided **Dunn's test if significant;** Otherwise **ANOVA** on log transformed data followed by **Dunnett test (2-sided) if significant.**
Urinary pH	**Kruskal-Wallis test**	No further analysis	2-sided **Dunn test**

C. METHODS

1. In-life observations including Functional Observational Battery (FOB)

Animals were observed twice daily for mortality and moribundity (once daily on weekends/public holidays) and at least once daily for clinical signs of toxicity. Detailed physical examinations were undertaken at least weekly, and the nature, onset, severity, duration and recovery of clinical signs were recorded. Cages and cage trays were examined daily for evidence of ill health.

During week 11 of the study, all animals were subjected to detailed neurological examinations undertaken by an examiner blinded to the dosing regimen of each animal. These examinations included a Functional Observational Battery examination and exploratory locomotor activity components. Further details regarding neurological examination are described below:**.**

Home cage observations:

Posture, piloerection, involuntary motor movements, gait abnormalities, vocalisations, abnormal behaviour.

Handling observations:

Ease of removal from cage, reaction to being handled, muscle tone, eyelid, lacrimation, salivation, nasal discharge, staining or any other signs such as alopecia, emaciation, temperature upon touching.

Open-field observations (individually for two minutes):

Piloerection, respiration, arousal, gait abnormalities, posture, involuntary motor movements, stereotypic movements, vocalizations and number of rearings, urine and faeces spots

Reflex and physiologic observations/measurements:

- Pupil size
- Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary constriction by focusing a narrow beam of light in the eyes).
- Surface righting reflex (by putting the animal on its back and evaluating its ability/rapidity to reassume a normal standing position).
- Corneal reflex (by touching the medial canthus with a fine object and observing the quick and complete closure of the eyelids).
- Flexor reflex (by pinching the toes and evaluating the presence/strength of the flexor response of each hindlimb).
- Auditory startle response (by evaluating the animal response to an auditory stimulus).
- Tail pinch response (by pinching the tail with a forceps and evaluating the animal reaction).
- Grip strength: the fore- and hindlimb grip strength of animals was measured quantitatively using a grip strength apparatus equipped with one pull and one push strain gauge (Bioseb, Chaville, France).
- Landing foot splay: the animal was dropped from approximately 30 cm above the surface and hindlimb foot splay was marked, measured and recorded.
- Body weight
- Rectal temperature

Exploratory locomotor activity:

Animals were tested individually in an automated photocell apparatus (Imetronic, Bordeaux, France) that recorded spontaneous exploratory locomotor activity in the novel environment over 60 minutes. Data were collected at regular intervals throughout this period.

2. Bodyweights

   All animals were weighed twice during acclimation, once at study initiation, on a weekly basis thereafter, and once prior to sacrifice.

3. Food consumption, food efficiency and daily intake

   Food consumption was recorded on a twice weekly basis for the first six weeks and then weekly thereafter, with any instances of food spillage recorded. This data together with bodyweights were used to estimate mean daily feed and test substance intakes.

   The mean achieved test substance intake was defined using the following formula:

$$\text{intake (mg/kg bw/d)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean bodyweight at end of selected interval (g)}}$$

   Estimates of food use efficiency were not provided and they were not estimated by the evaluator.

4. Ophthalmological examinations

   All animals during the acclimation period, and surviving animals from the control and 6000 ppm groups at study week 12 were subjected to ophthalmological examination by instillation of an atropinic agent.

5. Clinical pathology (haematology, clinical chemistry, coagulation, urinalysis)

   On study days 93 to 95, blood samples were drawn from the retro-orbital venous plexus of all animals after overnight fast under isoflurane anaesthesia. Blood was collected into tubes containing EDTA (0.5 mL) for haematology, clot activator (1.1 mL) for clinical chemistry, or sodium citrate (0.9 mL) for coagulation parameter analyses.

   The following clinical pathology parameters were examined.

Haematology

The following parameters were measured using an Advia 120 analyser (Siemens, Eragny, France), except for prothrombin time, which was assessed using an ACL Elite Pro (Instrumentation Laboratory, Paris, France). Blood smears were prepared and stained using May-Grünwald-Giemsa method and were examined in the event of Advia 120 analysis results being abnormal.

Red blood cell count	Haematocrit
Leucocyte count	Platelet count
Hemoglobin	Leucocyte differential count
Reticulocyte count	Mean corpuscular (cell) volume
Mean corpuscular (cell) hemoglobin	Prothrombin time
Mean corpuscular (cell) hemoglobin concentration	

Microscopic blood smear examination (May-Grünwald-Giemsa method)

Clinical Chemistry

The following parameters were measured using an Advia 1650 (Siemens, Eragny, France).

aspartate aminotransferase	glucose
alanine aminotransferase	total protein
gamma-glutamyltransferase	albumin
alkaline phosphatase	triglycerides
total bilirubin	inorganic phosphorus
calcium	sodium
urea	chloride
creatinine	potassium
cholesterol	

Additionally, overnight urine samples were collected from all rats on study days 87 or 88, with equal numbers per group over the two days. Food and water were withheld during the overnight collection period. Quantitative urinalyses parameters were measured by a Clinitek 500 and Multistix dipsticks (Siemens, Eragny, France). Refractive index was measured using a RFM320 refractometer (Bioblock Scientific, Illkirch, France). The following urinalysis parameters were analysed:

Appearance (colour, clarity)	
Occult blood/red blood cells	Ketones
Volume	Bilirubin
Specific gravity/osmolality/refractive index	pH
Glucose	Urobilinogen
Microscopic urine sediment examination	Protein

6. Sacrifice and pathology

All animals were subjected to necropsy, which included examination of all orifices, major organs, tissues and body cavities. Macroscopic abnormalities and masses were recorded and sampled, and if possible regional lymph nodes with potential involvement in macroscopic abnormalities and masses were also sampled. Samples were fixed by immersion in 10% neutral buffered formalin, with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were instead fixed in Davidson's fixative.

Liver, kidney, lung, stomach, and thyroid tissues from all groups were histopathologically examined. A standard suite of tissues (Table KIIA 5.8/06-3) from control and high dose animals were embedded in paraffin wax and subjected to histological analysis using haematoxylin and eosin staining.

Initial examinations of macroscopic and microscopic lesions by the study pathologist (M Fouque) was followed up with a review by an in-house review pathologist (F Schorsch) conducted in accordance with standard operating procedures. Final diagnoses were made as a consensus between the study pathologist and review pathologist.

Organs that were weighed and examined are listed in Table KIIA 5.8/06-3. Organ weight/final bodyweight and organ weight/brain weight ratios were calculated.

Table KIIA 5.8/06-3: Organs/tissues collected for pathological examination

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted[a]
Adrenal gland	X	X	X
Articular surface (femorotibial joint)	-	X	X
Aorta	-	X	X
Bone with bone marrow, sternum	-	X	X
Bone marrow smear[(B)]	-	X	-
Brain	X	X	X
Epididymis	X	X	X
Esophagus	-	X	X
Eye (with optic nerve)	-	X	X
Harderian gland	-	X	X
Heart	X	X	X
Kidney	X	X	X
Intestines (duodenum, jejunum, ileum, cecum, colon, rectum)	-	X	X
Lacrymal exorbital gland	-	X	-
Larynx/pharynx	-	X	-
Liver	X	X	X
Lung	-	X	X
Lymph nodes (submaxillary, mesenteric)	-	X	X
Mammary gland	-	X	X
Nasal cavities	-	X	-
Ovary	X	X	X
Pancreas	-	X	X
Parathyroid gland	X	X	X
Pituitary	X	X	X
Prostate	X	X	X
Salivary gland, submaxillary	-	X	X
Sciatic nerve	-	X	X
Skeletal muscle	-	X	X
Seminal vesicle		X	X
Skin	-	X	X
Spinal cord, (cervical, lumbar, thoracic)	-	X	X
Spleen	X	X	X
Stomach	-	X	X
Testis	X	X	X
Thymus	X	X	X
Thyroid gland	X	X	X
Tongue	-	X	X
Trachea	-	X	X
Urinary bladder	-	X	X
Uterus with cervix	X	X	X
Vagina	-	X	X
Gross lesions	-	X	X

Histopathological analysis of selected tissues was undertaken for main study control and high dose groups only, with the exception of suspect target organs (liver, kidney, stomach. lung, and thyroid) and macroscopic lesions which were processed to slides and examined microscopically for all dosing groups. 'B' – a bone marrow smear of one femur was prepared for each animal and stained by the May-Grűnwald-Giemsa method, but were not examined for any group due to the absence of abnormal haematology or bone marrow findings. Data excerpted from page 28 of the study report.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

No test substance-related clinical signs were observed. The clinical signs noted did not exhibit a dose response and no statistically significant increase in incidence was observed.

2. Mortality

There were no instances of unscheduled mortality during the study.

B. BODY WEIGHT AND BODY WEIGHT GAIN

There were no instances of group mean bodyweight loss over the weighing intervals. Effects on bodyweight gain were confined to 1000 ppm and above in both sexes.

In males at 1000 ppm, mean bodyweight by study day 8 was 4% lower than controls, 9% ($p \leq 0.01$) lower than controls by study day 29, and terminal bodyweights were 12% ($p \leq 0.01$) lower than controls at study day 92. Lower mean bodyweight and decreased bodyweight gain were also noted at 6000 ppm, with a mean bodyweight 4% lower than controls by study day 29, 6% lower by study day 57, with no further decreases through to study termination (all values were not statistically significant). Relative to controls, the extent of lower terminal bodyweight at 1000 ppm in males was 2-fold greater than that experienced by males at 6000 ppm. However, a dose-related response was not observed.

In females at 1000 ppm, mean bodyweight was 5% lower than controls at study day 57, and were 4% lower than controls by study day 92 (no statistical significance observed). Lower mean bodyweights during the treatment period were also observed at 6000 ppm in females, with a mean bodyweight 6% ($p \leq 0.05$) lower than controls at study day 29, 7% ($p \leq 0.05$) lower at study day 57 ($p \leq 0.05$), and 9% ($p \leq 0.01$) lower at study day 92 (Table KIIA 5.8/06-4)

Table KIIA 5.8/06-4: Bodyweights and cumulative bodyweight gains in animals administered BCS-AA56716

	Dose (ppm)							
	M				F			
	0	200	1000	6000	0	200	1000	6000
Initial BW (D 1)	194.6±6.6	196.1±6.1	194.8±7.2	196.3±6.2	162.1±7.9	163.6±7.1	161.4±6.9	162.4±7.0
BW Week 2 (D 8)	257.3±10.6	259.3±10.0 **(+1%)**	246.6±11.6 **(-4%)**	255.3±8.5 **(-1%)**	188.1±11.0	189.3±10.9 **(+1%)**	188.3±7.2 **(0%)**	184.1±7.8 **(-2%)**
BW Week 4 (D 29)	380.4±24.9	379.3±21.5 **(0%)**	347.5±23.6** **(-9%)**	363.8±19.5 **(-4%)**	240.1±14.5	241.8±13.5 **(+1%)**	235.2±12.0 **(-2%)**	225.4±10.4* **(-6%)**
BW Week 8 (D 57)	464.6±43.8	472.4±28.5 **(+2%)**	408.0±28.5** **(-12%)**	434.8±35.8 **(-6%)**	273.7±16.4	275.8±13.7 **(+1%)**	259.9±15.0 **(-5%)**	254.9±7.6* **(-7%)**
BW Week 13 (D 92)	511.7±48.3	530.6±26.6 **(+4%)**	449.6±30.1** **(-12%)**	479.3±43.4 **(-6%)**	294.9±19.0	294.6±16.2 **(0%)**	282.7±18.3 **(-4%)**	268.0±6.7** **(-9%)**
BWG Wks 1-4 (D 1 to 29)	185.8±21.9	183.2±17.6 **(-3%)**	152.7±20.0** **(-18%)**	167.6±19.5 **(-10%)**	78.1±9.8	78.1±10.2 **(0%)**	73.8±9.5 **(-6%)**	63.0±7.9** **(-19%)**
BWG Wks 1-8 (D 1 to 57)	270.0±41.5	276.3±24.9 **(+2%)**	213.2±25.1** **(-21%)**	238.5±34.4 **(-12%)**	111.6±13.1	112.1±10.8 **(0%)**	98.5±12.3* **(-12%)**	92.5±7.1** **(-17%)**
BWG Wks 1-13 (D 1-92)	317.1±47.3	334.5±23.2 **(+5%)**	254.9±26.2** **(-20%)**	283.0±42.2 **(-11%)**	132.8±18.6	131.0±11.6 **(-1%)**	121.4±16.0 **(-9%)**	105.7±7.0** **(-20%)**

* - Denotes statistical significance at the p≤0.05 or ** p≤0.01 level. Data excerpted from pages 32 and 57–76 of the study report.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

Decreased bodyweight gains in both sexes at 1000 or 6000 ppm (Table KIIA 5.8/06-5) was accompanied by corresponding decreases in absolute (g/d) food consumption from study day 4 onwards (earliest time point examined). At 1000 ppm, mean absolute food consumption (g) over the study was 6/4% (M/F) lower than controls, with peak decreases of 7/10% (M/F) at days 43 and 50 (M/F respectively). At 6000 ppm, mean absolute food consumption over the study was 5/7% (M/F) lower than controls, with peak decreases of 9/13% (M/F) at days 45 and 50 (M/F respectively). Estimates of food use efficiency were not provided in the study report and were not calculated by the evaluator.

Table KIIA 5.8/06-5: Food consumption (g/d)

	Dose (ppm)							
	M				F			
study day	0	200	1000	6000	0	200	1000	6000
4	24.97±0.712	25.5±0.441 (+2%)	22.13±0.542 (-11%)	23.43±0.441 (-6%)	18.43±0.78	18.33±0.678 (-1%)	17.47±0.203 (-5%)	16.87±0.271 (-8%)
8	26.35±0.608	26.78±1.139 (+2%)	24.8±0.456 (-6%)	26.5±0.557 (+1%)	19.63±0.937	19.68±0.734 (0%)	19.1±0.253 (-3%)	18.7±0.101 (-5%)
11	26.4±0.136	27.57±0.915 (+4%)	25±0.068 (-5%)	27.2±0.068 (+3%)	19.87±1.356	20.3±0.78 (+2%)	19.23±0.373 (-3%)	18.63±0.712 (-6%)
15	27.43±0.177	28.35±1.063 (+3%)	26.5±0.456 (-3%)	27.08±0.836 (-1%)	20.38±0.734	20.93±0.532 (+3%)	19.38±0.582 (-5%)	18.65±1.013 (-8%)
18	27.3±0.576	27.4±0.814 (0%)	25.73±0.271 (-6%)	26.23±1.39 (-4%)	21±0.542	20.53±0.61 (-2%)	19.63±0.576 (-7%)	18.37±1.254 (-13%)
22	27.38±0.836	27.58±0.633 (+1%)	25.75±0.506 (-6%)	26.08±0.734 (-5%)	20.65±1.114	20.68±0.076 (0%)	19.73±0.025 (-4%)	18.78±1.19 (-9%)
25	26.7±0.915	27.43±1.051 (+3%)	25.57±0.712 (-4%)	25.43±0.509 (-5%)	19.37±0.848	19.43±0.237 (0%)	19.07±0.475 (-2%)	18.17±0.576 (-6%)
29	26.9±1.215	27.25±1.063 (+1%)	25.4±0.608 (-6%)	24.95±0.911 (-7%)	20.15±0.557	20.08±0.532 (0%)	20±0.051 (-1%)	18.73±1.241 (-7%)
32	26.53±0.61	25.7±0.034 (-3%)	24.9±0.509 (-6%)	24.23±1.932 (-9%)	20±0.678	20.3±0.034 (+2%)	19.23±0.305 (-4%)	19±0.271 (-5%)
36	26.65±0.152	26.68±0.329 (0%)	24.25±1.317 (-9%)	24.48±1.747 (-8%)	20.83±0.481	20.2±0.709 (-3%)	19.55±0.253 (-6%)	19.55±0.253 (-6%)
39	26.97±2.407	26.17±0.509 (-3%)	23.67±1.153 (-12%)	23.87±1.492 (-11%)	19.07±0.475	19.4±1.221 (+2%)	18.63±0.17 (-2%)	18±0.407 (-6%)
43	26.75±0.911	26.63±0.633 (0%)	24.95±1.013 (-7%)	24.38±1.595 (-9%)	21.18±0.228	20.55±0.506 (-3%)	19.6±0.152 (-7%)	19.85±0.203 (-6%)
50	25.5±0.647	25.67±0.216 (+1%)	24.69±0.892 (-3%)	23.89±1.41 (-6%)	21.5±1.77	20.49±0.029 (-5%)	19.29±0.288 (-10%)	18.64±0.906 (-13%)
57	26.1±0.791	26.56±0.388 (+2%)	24.79±0.532 (-5%)	25.04±1.108 (-4%)	20.81±0.878	20.91±0.547 (0%)	19.96±0.59 (-4%)	19.77±0.46 (-5%)
64	24.96±1.165	25.97±0.863 (+4%)	23.21±0.561 (-7%)	23.79±1.424 (-5%)	20.36±0.475	19.46±1.094 (-4%)	19.4±0.259 (-5%)	18.84±0.187 (-7%)
71	25.01±0.705	25.61±0.187 (+2%)	24.17±0.144 (-3%)	24.29±1.122 (-3%)	19.66±0	19.76±0.791 (+1%)	19.86±0.201 (+1%)	19.43±0.46 (-1%)
78	24.63±0.46	24.61±0.705 (0%)	23.46±0.173 (-5%)	23.19±1.022 (-6%)	18.96±0.532	19.19±0.647 (+1%)	18.41±0.072 (-3%)	17.99±0.014 (-5%)
85	25.34±0.748	25.99±0.59 (+3%)	24.21±0.187 (-4%)	24.56±0.878 (-3%)	19.53±0.647	19.8±0.518 (+1%)	19.8±0 (+1%)	18.36±0.187 (-6%)
92	23.21±0.187	24.23±0.201 (+4%)	23.07±1.05 (-1%)	22.5±0.245 (-3%)	18.33±0.734	17.89±0.691 (-2%)	18.26±0.058 (0%)	17.66±0.115 (-4%)

1-92 $\bar{x}^A$	**26.1**	**26.4 (+1%)**	**24.5 (-6%)**	**24.8 (-5%)**	**20.0**	**19.9 (0%)**	**19.2 (-4%)**	**18.6 (-7%)**

'A' – calculated by the evaluator using Microsoft Excel. Statistical analyses of food consumption were not provided by the study author and were not conducted by the evaluator. Data excerpted from pages 78 to 85, with the exceptions of per cent change from control and mean consumption over study days 1 to 92, which were calculated by the evaluator using Microsoft Excel.

D. OPHTHALMOLOGICAL EXAMINATIONS

Ophthalmology findings were unremarkable.

E. CLINICAL PATHOLOGY

1. Haematology

Abnormal haematology findings were confined to females at 1000 and 6000 ppm only (Table KIIA 5.8/06-6), with a 9% lower haemoglobin, 6% lower mean corpuscular volume, and 8% lower mean corpuscular haemoglobin in females at 1000 ppm relative to controls. These findings all displayed poor dose dependency, with similar decreases reported at 6000 ppm. Small (2 to 3% lower) extents of decreases in these parameters were seen at 200 ppm, but obtained statistical significance only for MCH ($p \leq 0.05$).

Erythrocyte and reticulocyte counts in females were unaffected, as were all haematology parameters in males.

The study authors regarded the findings at 1000 ppm and 6000 ppm as test substance related and toxicologically adverse, but based on the small extent of change the decrease in MCH for females at 200 ppm to be test substance related but not toxicologically adverse. The evaluator agrees with these conclusions.

Table KIIA 5.8/06-6: Haematology findings in females

	F			
	0	**200**	**1000**	**6000**
Hb (g/dL)	15.89±0.608	15.43±0.387 **(-3%)**	14.43±0.670** **(-9%)**	14.60±0.440 **(-8%)**
MCV (fL)	54.6±1.25	53.6±1.28 **(-2%)**	51.3±0.80** **(-6%)**	52.1±0.85 **(-5%)**
MCH (pg)	17.76±0.519	17.23±0.427* **(-3%)**	16.35±0.387** **(-8%)**	16.58±0.391 **(-7%)**
Haematocrit (L/L)	0.4883±0.0153	0.4797±0.0107 **(-2%)**	0.4529±0.0206** **(-7%)**	0.4596±0.0158 **(-6%)**

Denotes statistical significance at the * $p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from page 34 of the study report.

2. Clinical chemistry

Changes in group mean clinical chemistry parameters were confined to decreased serum glucose and total bilirubin, and increased serum urea at all doses of the test substance (Table KIIA 5.8/06-7).

Serum glucose concentrations were 29/27% (M/F), 45/49%, and 45/53% lower than controls at 200, 1000, and 6000 ppm, respectively, being statistically significant at $p \leq 0.01$ level at each dose level. Total serum bilirubin was 27/12% (M/F), 52/37%, and 56/47% lower than

controls at 200, 1000, and 6000 ppm, respectively, and were statistically significant at p≤0.01 level except for the 200 ppm groups (p≤0.05 for M; not statistically significant for F).

The study authors regarded these findings to be test substance related but not adverse, as they did not represent any functional impairment in the animals. Noting the direction of change for the reported effects (decreases, rather than increases), the evaluator concurs with this conclusion.

Additionally, 14% (p≤0.01 for M; p≤0.05 for F) higher mean inorganic phosphorus concentrations in serum were seen at 6000 ppm. While a treatment-related change, toxicological adverseness is uncertain with the available data.

For urea concentrations, highest magnitude changes were seen in the 1000 ppm group for both sexes, with 15/14% (M/F) (p≤0.05), 26/23% (p≤0.01), and 18/12% (p≤0.05 for M; not statistically significant for F) increases from controls at 200, 1000, and 6000 ppm, respectively. Based on the absence of dose dependency at the high dose, and the absence of changes in creatinine concentration or histopathological correlates, the study authors did not regard the changes in urea to be test substance related. The study evaluator agrees with this conclusion.

Table KIIA 5.8/06-7: Select clinical chemistry parameters

	Dose (ppm)							
	M				F			
	0	200	1000	6000	0	200	1000	6000
Gluc (mmol/L)	5.68±0.533	4.00±0.556** **(-29%)**	3.14±0.393** **(-45%)**	3.12±0.245** **(-45%)**	6.04±0.490	4.42±0.544** **(-27%)**	3.07±0.587** **(-49%)**	2.82±0.321** **(-53%)**
Bili (µmol/L)	1.47±0.356	1.07±0.442[8] **(-27%)**	0.70±0.258** **(-52%)**	0.65±0.295** **(-56%)**	2.45±0.784	2.16±0.521 **(-12%)**	1.55±0.617** **(-37%)**	1.30±0.343** **(-47%)**
Urea (mmol/L	4.83±0.522	5.57±0.602* (**+15%)**	6.08±0.811** **(+26%)**	5.70±0.569 (**+18%)**	5.51±0.651	6.25±0.378 **(+14%)**	6.76±0.737** **(+23%)**	6.17±0.600** **(+12%)**

- Denotes statistical significance at the * p≤0.05 or ** p≤0.01 level. Data excerpted from page 35 of the study report, and reported means were rounded to two decimal places where applicable.

3. Urinalysis

Dose dependent increases in urinary volume were seen at all test substance doses in males, and in females at 1000 or 6000 ppm, but only attaining statistical significance in males at ≥1000 ppm (2.4-fold increase; p≤0.001) and in females at 6000 ppm (1.5-fold increase; p≤0.01). An increased incidence of higher ketone levels were seen in both sexes at 1000 ppm and above, and the study authors correlated this finding to decreased serum glucose levels in these groups (Table KIIA 5.8/06-8). The evaluator considers that the change in urinary volume and ketone levels are treatment-related, though the lack of other clinical pathology changes and histopathological effects suggests that the changes are unlikely to be toxicologically adverse in this case.

Additionally, urinary pH was decreased in males at 6000 ppm only, with a 0.5 pH unit decrease compared with controls. The study authors regarded this finding to not be adverse but did not provide further explanation. Overall, the evaluator considers that there is insufficient information to correlate pH changes with observed treatment-related changes, and no meaningful conclusion regarding toxicological adverseness can be reached with the available data.

Table KIIA 5.8/06-8: Select urinalysis parameters

		Dose (ppm)							
		M				F			
		0	200	1000	6000	0	200	1000	6000
Urine vol (mL)		3.50±1.08	7.43±1.730 **(+112%)**	11.9±5.731*** **(+240%)**	12.8±4.508*** **(+266%)**	1.66±1.727	2.13±1.29 **(+28%)**	3.41±1.56 **(+105%)**	4.24±1.62** **(+155%)**
Ketones (No. samples present per grade)	**Grade**								
	0	0	0	0	0	6	6	0	0
	1	7	2	0	0	1	2	0	0
	2	3	8	0	0	0	0	5	0
	3	0	0	0	0	0	0	4	7
	4	0	0	10	10	0	0	0	2
	Total	**10**	**10**	**10**	**10**	**7**	**8**	**9**	**9**

- Denotes statistical significance at the * $p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from page 36 of the study report.

F. FUNCTIONAL OBSERVATIONAL BATTERY

Functional observational battery findings were unremarkable. Minor variations from controls included increased frequency of weak tail pinch response (3/10 males at 6000 ppm), repetitive licking of lips (4/10 females at 6000 ppm) and stereotypic grooming (2/10 females at 6000 ppm), though the lack of treatment-related clinical signs and neurological effects suggested that the responses observed were incidental.

G. SACRIFICE AND PATHOLOGY

1. Organ weight

Organ weight data were unremarkable, with all changes being accounted for by decreased terminal bodyweights in test substance groups.

2. Gross pathology and histopathology

Test substance related findings at gross necropsy were confined to black foci in the stomach gland of both sexes at 1000 ppm (10/20% incidence for M/F) and 6000 ppm (30%/20% incidence for M/F). The finding was absent in control males but present in 1/10 control females (Table KIIA 5.8/06-9).

The study authors regarded this finding to be test substance related at 1000 ppm and 6000 ppm in both sexes, and 'mostly' associated at the histopathological level with focal glandular erosion/necrosis in the stomach. The evaluator agrees with the study author's conclusions.

All other histopathological findings were unremarkable.

Table KIIA 5.8/06-9: Gross necropsy and histopathology findings in stomach glandular region

	Dose (ppm)							
	M				F			
	0	200	1000	6000	0	200	1000	6000
Black focus(i) (macroscopic)	0/10	0/10	**1/10**	**3/10**	**1/10**	0/10	**2/10**	**2/10**
Erosion/necrosis, focal								
minimal	0	0	1	1	0	0	0	0
slight	0	0	0	1	0	0	0	1
moderate	0	0	0	0	0	0	1	0
total	**0**	**0**	**1**	**2**	**0**	**0**	**1**	**1**

Statistical analyses for these data were not undertaken. Data excerpted from page 37 of the study report.

The study author regarded the NOAEL (No Observed Adverse Effect Level) to be 200 ppm in both sexes (12.7/15.6 mg/kg bw/d for M/F). However, no specific discussion regarding the LOAEL of 1000 ppm (66.2/78.7 mg/kg bw/d for M/F) was provided. From the presented data, toxicologically adverse effects at 1000 ppm included decreased bodyweight gains in males, resulting in 12% lower terminal bodyweights relative to controls ($p \leq 0.01$), associated decreases in food consumption, black foci in the glandular part of the stomach (10/20% incidence for M/F) for both sexes that correlated histopathologically with focal glandular erosion/necrosis, and (in females only) statistically significant ($p \leq 0.01$) decreases in plasma haemoglobin (-9%), mean corpuscular volume (-6%), mean corpuscular haemoglobin (-8%) and haematocrit (-7%). The evaluator considers these findings at 1000 ppm to be sufficient basis for a LOAEL in this study.

III. CONCLUSION

Under the conditions of this study BCS-AA56716 did not produced test substance-related effects on survival, clinical signs, FOB parameters, ophthalmological observations, or organ weights.

The No Observed Adverse Effect Level (NOAEL) in this study is 200 ppm for both sexes (12.7/15.6 mg/kg bw/d for M/F). The LOAEL was 1000 ppm (66.2/78.7 mg/kg bw/d for M/F), based on decreased bodyweight gains in males, resulting in 12% lower terminal bodyweights relative to controls ($p \leq 0.01$), associated decreases in food consumption, black foci in the glandular part of the stomach (10/20% incidence for M/F) for both sexes that correlated histopathologically with focal glandular erosion/necrosis, and (in females only) statistically significant ($p \leq 0.01$) decreases in plasma haemoglobin (-9%), mean corpuscular volume (-6%), mean corpuscular haemoglobin (-8%) and haematocrit (-7%).

This study is classified as fully reliable (acceptable/guideline) and satisfies guideline requirements for a subchronic oral toxicity in rats [OPPTS 870.3100; OECD 408 (1998)].

Primary review: Australia
Secondary review: US EPA & PMRA

Report: Eigenberg DA (2010) A 90-Day Toxicity Feeding Study in the Beagle Dog with Technical Grade BYI 02960. Xenometrics, LLC 17745 Metcalf Ave., Stilwell, Kansas 66085. Study No**.:** 09-S76-QQ. 22 April 2010. MRID 48844114. PMRA 2239504. Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor report number: M-369978-01-2.

Guidelines: OPPTS 870.3150 (1998)
OECD 409 (1998)

Deviations: Minor animal housing deviations were not considered to affect study conclusions.

GLP: Yes Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a 90-day dietary study (MRID 48844114), BYI 02960 was administered to seven to eight month old beagle dogs in groups of 4/sex/dose at dietary concentrations of 0, 400, 1200, or 3600/2400ppm (high dose reduced from 3600 ppm starting on study week 9). Dose levels were equivalent to 0/0, 12/12, 33/41, and 102[85]/107[78] mg/kg bw/d respectively.

BYI 02960 did not affect mortality, ophthalmology, or urinalysis parameters in this study.

Clinical signs were confined to the 3600 ppm dose groups, where one male and one female experienced unsteady/stiff back legs and lower back on study days 44 (both sexes) and 53 and 54 (for the male only). These findings led to reduction of the dose to 2400 ppm from study week 9 (day 56) onwards.

Over study days 0 to 14, both sexes at 3600 ppm experienced group mean bodyweight loss of 4/6% (M/F), respectively. Mean bodyweights at 3600/2400 ppm were over 10% lower than controls by study week 7 (11/14% decrease in M/F, statistically insignificant), reached a maximum difference of 14/16% ($p \leq 0.05$ in M only) lower than controls at study week 8, and partially recovered thereafter, resulting in terminal bodyweights 11/13% lower than controls at study week 13. Group mean bodyweight gains in both sexes at 1200 ppm gradually decreased from controls from study week 3 onwards, resulting in 9/7% lower body weights compared with controls at study week 13. The decreases in bodyweight gain as well as instances of bodyweight loss were at least partially accounted for decreased food consumption.

In haematology, dose- and time point-related trends were observed for decreases in RBC count, plasma haemoglobin and haematocrit compared with controls, and the findings are consistent with a treatment-related effect at 3600/2400 ppm.

In clinical chemistry, both sexes at ≥ 1200 ppm had marked increases in group mean creatine kinase (CK), aspartate aminotransferase (AST), and alanine aminotransferase activities (ALT), and at 3600/2400 ppm there were decreased serum creatinine levels

and increased serum cholesterol in both sexes, and increased glucose in females. However, all findings but ALT activity in males at ≥1200 ppm had resolved by study day 84.

In terminal findings, organ weight changes were observed in 3600/2400 ppm males, with relative (to bodyweight) kidney weights increased by 31% ($p \leq 0.05$), absolute prostate weights increased by 67% ($p \leq 0.05$) and relative prostate weights by 88% ($p \leq 0.05$).Increased relative kidney weights (16%; not statistically significant) were also observed in females at 3600/2400 ppm, and increased absolute (14/11% in M/F, $p \leq 0.05$ in M only) and relative (28/24% in M/F, $p \leq 0.05$) liver weights were also seen at 3600/2400 ppm.

Pathology examinations reported skeletal muscle myofiber atrophy/degeneration in 2/4 males (both minimal severity) and all four females (3 minimal, 1 slight severity) at 1200 ppm, with an increase in severity at 3600/2400 ppm in males only (1 minimal, 1 slight). All four females at 3600/2400 ppm reported skeletal muscle myofiber atrophy/degeneration of minimal severity. Accumulation of brown pigment to a minimal severity was seen in hepatic Kupffer cells of 2/4 females at 3600/2400 ppm, but was absent from all other groups.

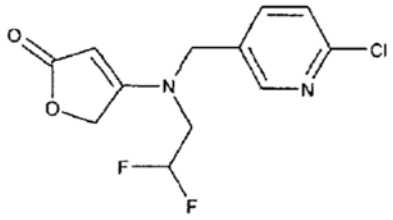
The no-observed-adverse-effect level (NOAEL) for both sexes was 400 ppm (12 mg/kg/day). The LOAEL was 1200 ppm (33 mg/kg/day), based on increased incidence of skeletal muscle myofiber atrophy/degeneration with associated increases in CK. AST, and ALT levels.

This study is classified as fully reliable (acceptable/guideline) and satisfies guideline requirements for a subchronic oral toxicity in dogs [OPPTS 870.3150; OECD 409].

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960



Batch #:	BYI 02960-01-03
Origin batch #:	2009-000239
Purity:	96.2%
Description:	Beige powder
CAS #	Not reported

Stability of test compound:	Stated to be stable for two years if stored at 25±5ºC (date of analysis 16 January 2009). Batches prepared on weeks 1, 2, 3, 6, 11, and 14 were subjected to analyses of achieved test substance concentrations and homogeneity, as well as stability analysis after storage at -18°C. Concentrations and homogeneity were reported to be 99 –103% of nominal values.
2. Vehicle:	Corn oil and acetone used to add the test material into diet.
3. Test animals	
Species:	Dogs
Strain:	Beagle
Age at dosing:	7 to 8 months
Weight at dosing:	7.9 to 9.8 kg (males), 6.0 to 7.7 kg (females)
Source:	Marshall BioResources, North Rose, New-York, USA
Acclimation period:	11 days
Diet:	Purina Mills Lab Canine Diet Etts 5006-3 *ad libitum*.
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Individually in stainless steel runs.
4. Environmental conditions	
Temperature:	18 to 29°C
Humidity:	30 to 70%
Air changes:	Approx. 17 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Initiated/completed (in-life phase)

   14 April – 15 July 2009

2. Animal assignment and treatment

   Details of animals and dosing levels are shown in Table KIIA 5.3.3/01-1. Four groups of 4 dogs/sex/dose received concentrations of 0, 400, 1200, or 2400/(reduced from 3600 ppm starting on study week 9) BYI 02960 in portions of feed that were replaced daily over 91 or 92 days. Animals were then fasted overnight, sacrificed, and subjected to necropsy.

   The initial dosing regimen was based on results from two dietary administration studies in dogs. In the first study (Kennel, 2008; Study No. SA 07291) dosing at 5000 ppm for 14 days resulted in decreased food consumption and bodyweight loss (< 10%). In the second study (Odin-Feurtet, 2008; Study No. SA 07290), dosing at 4000 ppm for 28 days resulted in decreased body weight gain, decreased food consumption, increased platelet counts and decreased centrilobular liver glycogen.

   The high dose was reduced from 3600 ppm to 2400 ppm on day 56 after two dogs exhibited clinical signs and continued bodyweight loss from study day 44 until day 56.

Achieved mean daily test substance intakes at 0, 400, 1200, and 3600(2400) ppm were 0/0, 12/12, 31/41, and 102(85)/107(78) mg/kg bw/d for M/F, respectively [intakes at the high dosing regimen after study week 9 shown in parentheses].

Animals were randomised by sex to dosing groups using computer software (Instem Datatox®) such that bodyweights in any one group deviated by less than 20% from the group mean.

Table KIIA 5.3.3/01-1: Study design

nominal conc. in diet (ppm)	**achieved mean daily intake (mg/kg bw/d)[A]**		**animal ID No's [N=4]**	
	M	F	M	F
0	0	0	QQ0001 to 0004	QQ0101 to 0104
400	12	12	QQ1001 to 1004	QQ1101 to 1104
1200	33	41	QQ2001 to 2004	QQ2101 to 2104
3600 (wks 1-8) 2400 (wks 9 to 12)	102/85	107/78	QQ3001 to 3004	QQ3101 to 3104

[A]- Estimates of exposure were based on measured concentrations of test substance in feed and measured feed intakes. Data excerpted from pages 13 of the study report.

3. Diet preparation and analysis

The required amount of the test material suspended in volumes of corn oil (1% w/w of diet) and acetone vehicle was incorporated into the diet by mixing to achieve the nominal dietary concentrations at each dosing level. The formulations were then stored under freezer conditions (-18°C) until the day of presentation to animals.

The concentration of BYI 02960 in the diet after freezer storage was determined on weeks 1, 2, 3, 6, 11, and 14, and ranged from 99-103% of nominal level. In a separate study (Moore, 2009; Study No. TXRVP010A), the test material in the diet at 100 and 5000 ppm was found to be homogenous (96–100% of nominal levels; relative standard deviations for sample from the top, middle and bottom layers of 3.8-3.9%) and stable for 7 days at room temperature (100–104% of nominal levels) as well as being stable for 35 days at freezer temperature (95–102% of nominal levels).

4. Statistics

Statistical analyses were conducted using INSTEM DATATOX® software, and 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 that were one-tailed (Table KIIA 5.3.3/01-2).

Table KIIA 5.3.3/01-2. Statistics used in data analyses

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant ($p>0.05$)	**If preliminary test is significant ($p\leq0.05$)**
Continuous data: Terminal bodyweight Bodyweight gain Clinical chemistry Organ weight (absolute & relative[A])	**Bartlett Test**	Analysis of variance followed by **Student's t-test if significant**	**Kruskal-Wallis test** followed by **Mann-Whitney U-test if significant**
Frequency data: Necropsy findings	**Chi-square test** followed by **Fisher's Exact test if significant.**		

C. METHODS

1. Observations

   Observations of clinical signs were undertaken twice daily (mornings and afternoons/evenings) except for weekends and public holidays where observations were made daily. Detailed physical observations were undertaken seven days prior to dosing initiation and on a weekly basis thereafter.

2. Body weights

   All animals were weighed at study initiation and at necropsy, and on a weekly basis over the study period.

3. Food consumption, food efficiency, and daily intake

   Food consumption was recorded daily during the main treatment period up to sacrifice on study days 92 or 93. Food use efficiency estimates were not provided.

4. Clinical pathology (haematology, coagulation, and clinical chemistry)

   Haematology and clinical chemistry evaluations were performed on fasted animals once prior to initiation of dosing and during study weeks 5, 9, and 13. Blood was collected by puncturing the jugular vein.

   The following parameters were examined:

Haematology and coagulation

Red blood cell count	Hemoglobin
Reticulocyte count	Hematocrit
Platelet count	Mean corpuscular (cell) volume
White blood cell count	Prothrombin time
Differential white blood cell count	Blood cell morphology
Mean corpuscular haemoglobin	
Activated partial thromboplastin time	
Mean corpuscular (cell) hemoglobin concentration	
Red blood cell distribution width	Haemoglobin distribution width

Clinical chemistry parameters

aspartate aminotransferase	total protein (in serum)
alanine aminotransferase	albumin
alkaline phosphatase	total bilirubin
creatinine phosphokinase	lactic acid dehydrogenase
urea nitrogen	creatinine
total cholesterol	triglycerides
glucose	chloride
sodium	potassium
calcium	inorganic phosphorous
gamma-glutamyltransferase	globulins and A/G ratio
uric acid	

5. Urinalysis parameters

Urine collected from all animals pre-exposure and during weeks 5, 9, and 13 was analysed for the following parameters:

appearance	ketone
24 hour urine volume	protein
specific gravity/osmolality	bilirubin
pH	blood
sediment	urobilinogen
glucose	leukocytes
nitrite	

6. Ophthalmology

Ophthalmological analyses of eyes from all animals were performed during acclimation and at study day 86 using a mydriatic agent. Pupillary reflexes were checked with a penlight or a transilluminator with Finnoff (Welch Allyn, Inc., Skaneateles Falls, NY). The conjunctiva, cornea, and lens were examined using a slit lamp microscope KOWA SL-15 (Kowa Company, Ltd, Tollerance, CA, USA). The vitreous humor, retina, choroid, and optic disc were examined with an indirect ophthalmoscope HEINE Omega (Heine USA Ltd, NH) and condensing lenses. Examinations were performed in a semi-darkened room.

7. Sacrifice and pathology

On study days 91 or 92, all animals were sacrificed by intravenous injection of Fatal-Plus® (Vortech Pharmaceuticals, Dearborn, Michigan) and subjected to gross necropsy. Gross necropsy included examination of each animal's general physical condition, body orifices, external and internal organs and tissues. Absolute and relative weights of major organs were determined. All tissues from control and high-dose (3600/2400 ppm) groups were processed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under a light microscope (**Table KIIA 5.3.3/01-3)**. Tissues collected from all animals in the control and high dose animals were subjected to histological analysis. If tissues in the high dose group were found upon histological analysis to have potential treatment-related abnormalities, they were also examined histologically in the low and mid dose groups. The liver and muscle tissue were examined for all animals.

Samples (including macroscopic abnormalities) were recorded and fixed by immersion in 10% neutral buffered formalin, except for the eyes and optic nerve that were preserved in Davidson's fixative, and ovaries, epididymis and testis that were preserved by Bouin’s fixative. Selected organs including brain, adrenals, kidney, liver, uterus, thyroid and parathyroid were weighed and relative organ weights calculated.

Table KIIA 5.3.3/01-3: Organs/tissues collected for pathological examination

Tissue [N=4/sex for controls and high dose]	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted[a]
Adrenal gland	X	X	X
Aorta		X	X
Bone marrow		X	X
Bone (sternum)		X	X
Bone (rib/cc jct)		X	X
Brain (cerebrum, midbrain, cerebellum, medulla/pons)	X	X	X
Cervix		X	X
Epididymides	X	X	X
Eosophagus		X	X
Eye (with optic nerve)		X	X
Heart	X	X	X
Kidney	X	X	X
Intestines (duodenum, jejunum, ileum, cecum, colon, rectum)		X	X
Larynx/pharynx		X	X
Liver with gallbladder	X	X	X
Lung	X	X	X
Lymph nodes (retropharyngeal, mesenteric)		X	X
Mammary gland		X	X
Ovary	X	X	X
Oviduct		X	X
Pancreas		X	X
Pituitary	X	X	X
Prostate	X	X	X
Salivary glands		X	X
Sciatic nerve		X	X
Skeletal muscle		X	X
Skin		X	X
Spinal cord, (cervical, lumbar, thoracic)		X	X
Spleen	X	X	X
Stomach		X	X
Testis	X	X	X
Thymus	X	X	X
Thyroid with parathyroid	X	X	X
Trachea		X	X
Ureter		X	X
Urinary bladder		X	X
Uterus	X	X	X
Vagina		X	X
Gross lesions		X	X

[a] Due to processing error, ovaries in the high dose group were fixed in formalin instead of in Bouin's fixative.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

   Clinical signs were confined to the 3600 ppm dose groups. One male (QQ3002) and one female (QQ3101) experienced unsteady/stiff back legs and lower back on study days 44 (both sexes) and 53 and 54 (for the male only). This finding, together with bodyweight loss in this dose group, led to the study director decision to reduce the test substance concentration from 3600 ppm down to 2400 ppm from study week 9 (day 56) onwards.

2. Mortality

   There were no unscheduled mortalities.

B. BODY WEIGHT AND BODY WEIGHT GAIN

Over study days 0 to 14, both sexes at 3600 ppm experienced group mean bodyweight loss of 4/6% (M/F) of their initial bodyweight, respectively. Mean bodyweight gain in males at this dose occurred sporadically, resulting in a mean bodyweight loss over study weeks 0 to 13 of 4% of their initial bodyweight, while no mean bodyweight gains occurred in females (**Table KIIA 5.3.3/01-4)**. Mean bodyweights at 3600/2400 ppm were over 10% lower than controls by study week 7 (11/14% decrease in M/F, statistically insignificant), reaching a maximum difference of 14/16% ($p \leq 0.05$ in M only) lower than controls at study week 8. Partial recovery of body weights was noted thereafter, with terminal bodyweights 11/13% lower than controls at study week 13.

Additionally, group mean bodyweight gains in both sexes at 1200 ppm gradually decreased from controls from study week 3 onwards, resulting in mean terminal body weights that were 9/7% lower than controls at study week 13.

Overall, the evaluator considers the extent of decreased bodyweight gains in both sexes at $\geq$ 1200 ppm to be toxicologically adverse, noting the decreased bodyweight gain at $\geq$ 1200 ppm and body weight loss at 3600/2400 ppm. The evaluator also notes that the overall body weight gain at 400 ppm in females is clearly decreased compared with controls over the treatment period. However, the overall decrease in body weight gain is comparable to the decreased food consumption observed in the 400 ppm group, and noting the lack of treatment-related findings in clinical pathology and histopathology at this dose level, it is unlikely that the decreased body weight gain in this case is toxicologically adverse.

Table KIIA 5.3.3-01/4: Group mean bodyweights

	Bodyweight (g)							
	M				F			
	Dose (ppm)							
Study day	0	400	1200	3600/2400	0	400	1200	3600/2400
-7	8752.5±596	8687.3±293.6 (-1%)	8497.5±654.2 (-3%)	8840±590.9 (1%)	6456.8±463.1	6524±523.6 (1%)	6383.5±552.7 (-1%)	6317.8±720.7 (-2%)
0	9071.3±504.5	9045.5±393.1 (0%)	8880±754.7 (-2%)	9233±634.6 (2%)	6769.8±347.8	6957.8±646.9 (3%)	6842±634.5 (1%)	7020.8±693.3 (4%)
7	9260±591.4	9409.5±415.9 (2%)	8930.3±814.1 (-4%)	8981±722.5 (-3%)	7270.5±743.6	7098.3±745.1 (-2%)	6938.8±733.2 (-5%)	6815.5±669.8 (-6%)
14	9480.8±771.2	9631.8±399.2 (2%)	8919.3±908.7 (-6%)	8912.8±763.5 (-6%)	7122.3±902.4	7220.8±853.5 (1%)	7016.3±629.3 (-1%)	6632.5±799 (-7%)
21	9558±762.9	9685.5±362.9 (1%)	9000.5±957.6 (-6%)	9056.5±817.8 (-5%)	7507.8±931.8	7399.3±822.2 (-1%)	7082.3±551.8 (-6%)	6963.3±600.5 (-7%)
28	9355.5±741.8	9569.8±232.9 (2%)	8887.5±822.7 (-5%)	8875±621.8 (-5%)	7329.5±927.7	7272.5±881.7 (-1%)	7030.8±612.6 (-4%)	6784.5±586.7 (-7%)
35	9830.5±630.8	10027±213.5 (2%)	9251.8±729.8 (-6%)	9212.5±755.2 (-6%)	7823±833.1	7650.3±917.9 (-2%)	7423±787.9 (-5%)	7023±618.4 (-10%)
42	9711±649.4	9998.5±212.7 (3%)	9049.5±840 (-7%)	8893.3±800.4 (-8%)	7704.3±844.6	7666±994.3 (0%)	7295.8±684.8 (-5%)	7021.3±556.4 (-9%)
49	9737.8±253.2	10210.3±321.2 (5%)	9345±1050.3 (-4%)	8624.3±893.3 (-11%)	7895.5±612.7	7851.3±1026.9 (-1%)	7516.3±592.9 (-5%)	6820.3±467 (-14%)
56	9542.8±196.1	9977.3±283.5 (5%)*	8765.5±901.9 (-8%)	8230.3±984.5 (-14%)*	7733±650	7554.8±944.7 (-2%)	7395.3±800.7 (-4%)	6530.8±546.6 (-16%)
63	9881±439.8	10370±264.9 (5%)	9145.5±1001.3 (-7%)	8695±950.1 (-12%)*	8010.8±854.7	8015.8±1009.5 (0%)	7652.3±909.3 (-4%)	6951.5±710.1 (-13%)
70	9928.5±572.8	10428.3±344.8 (5%)	9142.5±965.6 (-8%)	8865.5±813.4 (-11%)	8231.3±883.6	8038.3±1041.3 (-2%)	7536.5±651.6 (-8%)	7100.8±662.2 (-14%)
77	10067±546.9	10592.8±407.8 (5%)	9285.8±873.8 (-8%)	9029.3±824.9 (-10%)	8355±932.4	8065.3±1004.9 (-3%)	7657.8±1067.1 (-8%)	7315±611.2 (-12%)
84	9847.8±462.8	10327.3±373.1 (5%)	8940±822.1 (-9%)	8812±951.4 (-11%)	8094.8±845.8	7721.3±876 (-5%)	7512.8±942.3 (-7%)	7021.5±654.4 (-13%)
BW Gain (0 to day 84)	776.6±327.9	1281.8±577.2 (65%)	60±206.2 (-92%)*	-421±386 (-154%)*	1325±511.9	774.8±397.9 (-42%)	670.8±411.1 (-49%)	0.8±276.9 (-100%)*

'*' - Denotes statistically significant at the p<0.05 level by two sided ANOVA and Student's t-tests. Note, from week 9 (day 56) onwards, dietary concentrations of 3200 ppm in feed were reduced to 2400 ppm for both males and females. Data excerpted from pages 30 to 33 of the study report, except for per cent changes from control which were calculated by the evaluator using Microsoft Excel and rounded to integers.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

Decreased food consumption was observed at ≥ 1200 ppm during the dosing period (Table KIIA 5.3.3/01-5). Decreased food consumption at study week 1 ranged from 9 to 42% lower mean intakes in males at ≥1200 ppm, and 18 to 60% lower intakes in females across all three dosing groups without clear dose dependency. Similarly, feed intake levels (in g/animal/day) from study weeks 2 to 8 partially recovered but remained lower than controls by up to 34% in both mid and high dose males and all test substance–treated females. Overall, the magnitude of lower food consumption was equivalent in males at the 1200 and 3600/2400 ppm groups, and equivalent for females across the three dosing groups.

It is likely that the decreases in bodyweight gain were associated with decreased food consumption, particularly during the first two weeks of the study, and decreased food use efficiency given that decreases in food intake were similar at 1200 ppm and 3600/2400 ppm.

Food use efficiency was not calculated by the study authors.

Table KIIA 5.3.3/01-5: Mean food consumption (grams/animal/day) over study days 1 to 7 and study weeks 2 to 13

Study week or (day) N=4	Mean food consumption (grams/animal/d)							
	M				F			
	Dose (ppm)							
	0	400	1200	3600/2400	0	400	1200	3600/2400
(day 1)	346.7±110.4	346.7±54.8 **(0%)**	231±143.7 **(-33%)**	264.5±137.5 **(-24%)**	344.2±116.0	214.2±57.6 **(-38%)**	214.5±60.0 **(-38%)**	162±75.0 **(-53%)**
(day 2)	335.7±67.3	346.2±22.2 **(3%)**	194.5±61.6* **(-42%)**	205±87.0* **(-39%)**	356±95.3	248.7±88.8 **(-30%)**	216.2±22.0 **(-39%)**	142.7±62.5 **(-60%)**
(day 3)	307.5±101.8	341.5±68.6 **(11%)**	207±84.9 **(-33%)**	184±10.5* **(-40%)**	345±109.3	227.7±32.7 **(-34%)**	237.7±51.1 **(-31%)**	152.7±21.6 **(-56%)**
(day 4)	314.7±81.4	336±75.18 **(7%)**	193.2±48.2 **(-39%)**	285.7±112.2 **(-9%)**	357.7±93.8	259.2±53.9 **(-28%)**	273.5±51.2 **(-24%)**	158.5±56.8 **(-56%)**
(day 5)	311.5±94.9	330.7±61.34 **(6%)**	226±29.9 **(-27%)**	199.2±59.7 **(-36%)**	348.7±116.6	209.7±56.1 **(-40%)**	286±109.2 **(-18%)**	194±36.5 **(-44%)**
(day 6)	347.7±73.4	335.7±46.8 **(-3%)**	203±96.2* **(-42%)**	223.2±45.3* **(-36%)**	313±147.6	235.3±57.7 **(-25%)**	217.2±107.0 **(-31%)**	188.2±61.8 **(-40%)**
(day 7)	321.7±76.9	332.7±91.6 **(3%)**	228.7±42.0 **(-29%)**	235.5±74.6 **(-27%)**	386.2±66.1	232.5±36.1 **(-40%)**	242±48.2 **(-37%)**	189±74.0 **(-51%)**
wk 2	292.9±262.9	348.4±157.7 **(19%)**	228.7±159.0 **(-22%)**	217.4±166.9 **(-26%)**	306.8±291.1	237.9±161.3 **(-22%)**	251.1±132.8 **(-18%)**	220.1±188.6 **(-28%)**
wk 3	268.7±168.1	296.1±310.3 **(10%)**	234.8±142.2 **(-13%)**	254.5±213.7 **(-5%)**	304.9±216.7	225.1±169.6 **(-26%)**	229.8±145.7 **(-25%)**	216.2±170.9 **(-29%)**
wk 4	271.4±179.7	282.8±223.6 **(4%)**	219.9±221.5 **(-19%)**	237.4±202.0 **(-13%)**	266.1±203.8	226.8±297.8 **(-15%)**	241.7±217.1 **(-9%)**	197.1±140.3 **(-26%)**
wk 5	295.9±196.3	334.6±187.9 **(13%)**	264.9±187.1 **(-10%)**	274.7±165.6 **(-7%)**	290.9±140.4	238.5±177.7 **(-18%)**	267.7±140.3 **(-8%)**	232.0±178.1 **(-20%)**

wk 6	285.5±147.7	302.1±200.6 **(6%)**	257.8±103.9 **(-10%)**	261.3±242.1 **(-8%)**	262.9±141.2	236.6±133.8 **(-10%)**	248.7±160.2 **(-5%)**	209.6±63.9 **(-20%)**
wk 7	292.2±181.5	306.6±200.3 **(5%)**	261.8±157.3 **(-10%)**	227.5±163.3 **(-22%)**	268.7±227.9	219.6±140.3 **(-18%)**	246.9±106.1 **(-8%)**	178.4±160.4 **(-34%)**
wk 8	264.8±251.3	271.5±188.1 **(3%)**	214.0±194.7 **(-19%)**	232.8±256.5 **(-12%)**	234.4±288.3	198.4±154.9 **(-15%)**	224.4±211.9 **(-4%)**	174.0±139.4 **(-26%)**
wk 9	335.7±211.0	312.6±193.6 **(-7%)**	281.6±197.0 **(-16%)**	346.0±260.9 **(3%)**	263.8±161.4	237.8±126.5 **(-10%)**	278.2±143.2 **(5%)**	251.4±172.8 **(-5%)**
wk 10	280.7±264.1	312.0±169.9 **(11%)**	273.7±209.2 **(-3%)**	313.0±230.6 **(11%)**	249.1±168.4	220.4±137.7 **(-12%)**	266.0±137.9 **(7%)**	248.8±126.8 **(0%)**
wk 11	261.9±147.4	284.3±188.5 **(9%)**	266.1±146.1 **(2%)**	291.5±195.8 **(11%)**	249.1±209.9	220.3±171.6 **(-12%)**	244.1±126.9 **(-2%)**	229.9±97.6 **(-8%)**
wk 12	278.7±256.4	301.0±302.7 **(8%)**	244.1±192.1 **(-12%)**	288.0±318.3 **(3%)**	231.8±327.4	181.9±278.0 **(-22%)**	210.7±247.1 **(-9%)**	210.9±206.2 **(-9%)**
wk 13	255.2±248.0	286.0±485.6 **(12%)**	266.3±305.0 **(4%)**	308.2±274.5 **(21%)**	251.6±239.5	178.4±205.0 **(-29%)**	221.7±199.9 **(-12%)**	202.0±230.2 **(-20%)**

'%' denotes per cent change from control. '*' - Denotes statistically significant at the $p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from pages 31 to 57, except for mean weekly consumption, standard deviations, and per cent change from control, which were calculated by the evaluator using Microsoft Excel. Group means were rounded to one decimal place. Standard deviation was calculated using the formula dX/X = sqrt((dA/A)2 + (dB/B)2 +...), where 'dX' is the SD of mean 'X'.

D. CLINICAL PATHOLOGY

1. Haematology and coagulation

Statistically significant ($p \leq 0.05$) changes in haematology parameters were observed at ≥ 1200 ppm in both sexes (Table KIIA 5.3.3/01-6).

A 9/8% (M/F) decrease in haemoglobin and a 10/8% (M/F) decrease in haematocrit (both $p \leq 0.05$ in M only) were noted on study day 28 in 3600/2400 ppm animals. A slightly decreased RBC count (-7% in both sexes) was also noted, which was not observed in pre-test values (which, for the assigned animals, was greater than control values).

On day 56, the decreases in haemoglobin (13/21% lower in control in M/F; $p \leq 0.05$) and haematocrit (14/18% lower in M/F; $p \leq 0.05$) at 3600/2400 ppm had increased in severity and reached statistical significance in both sexes. Furthermore, a 7% ($p \leq 0.05$) lower erythrocyte count in females was seen at 1200 ppm, reaching a 16% ($p \leq 0.05$) lower count at 3600/2400 ppm. A 9% lower erythrocyte count was seen in males at 3600/2400 ppm but without statistical significance.

On day 84, haemoglobin concentrations in serum of animals at 3600/2400 ppm remained decreased by 9/11% (M/F) without statistical significance, and haematocrit concentrations remained decreased to a similar extent (9/10% in M/F; $p \leq 0.05$ in M). The RBC count was decreased compared with controls (5/8% decrease), but not statistically significant.

In addition, reductions in mean corpuscular volume in males from the 1200 and 3600/2400 ppm dose groups were observed at study day 28 (4-5%; $p<0.05$), and study days 56 and 84 (3-5%; $p<0.05$ at 3600/2400 ppm only). Increases in haemoglobin distribution width were demonstrated at study day 28 in all male dose groups (8-17%; $p<0.05$) and at study day 84 in the 1200 and 3600/2400 ppm dose groups (9-13%; $p<0.05$) but not at study day 56. An increase of 6% in red cell distribution width ($p<0.05$) was limited to males from the 3600/2400 ppm dose group at study day 84. Dose-related changes in these parameters were not observed in females, but a transient decrease in mean corpuscular haemoglobin (5%; $p<0.0.05$) compared to control was observed in the 3600/2400 ppm dose group females at study day 56. Given the small magnitude of these changes and the transient nature of several of the changes, these findings are not considered to be toxicologically relevant.

Overall, dose- and time point-related trends were observed for decreases in RBC count, plasma haemoglobin and haematocrit, and overall the evaluator considers that the findings are consistent with a treatment-related effect at 3600/2400 ppm.

The study authors regarded test substance related decreases to have occurred in plasma red blood cells, haemoglobin, and haematocrit at the 28 day, 56 day, and 84 day haematology examination periods. They regarded the statistically significantly ($p\leq0.05$) decreased erythrocyte count in females at 1200 ppm to be unrelated to the test substance, based on the absence of concurrent decreases in haemoglobin or haematocrit concentrations at this dose. On the basis of an observable dose relationship and the proportional 7% decreases in haemoglobin and haematocrit levels at this dose (albeit without statistical significance at the $p<0.05$ level), the evaluator disagrees that 7% decreases in erythrocyte count in females at 1200 ppm on study day 56 was unrelated to the test substance. However, the effect was transient, having resolved by day 84 and is therefore is not considered to be toxicologically adverse.

Table KIIA 5.3.3/01-6: Selected haematology parameters at study days 28, 56, and 84

	M				**F**			
	Dose (ppm)							
	0	**400**	**1200**	**3600/2400**	**0**	**400**	**1200**	**3600/2400**
Pre-test								
Hb (g/dL)	16.3 ± 1.3	17.1 ± 0.8 **(+5%)**	15.7 ± 0.8 **(-4%)**	16.8 ± 0.5 **(+3%)**	15.7 ± 0.7	15.9 ± 0.9 **(+1%)**	15.7 ± 0.6 **(0%)**	16.5 ± 0.5 **(+5%)**
Hct (%)	47.5 ± 3.7	50.0 ± 3.2 **(+5%)**	45.5 ± 2.1 **(-4%)**	49.6 ± 1.4 **(+4%)**	45.5 ± 2.4	46.0 ± 2.6 **(+1%)**	44.6 ± 0.9 **(-2%)**	47.6 ± 1.3 **(+5%)**
RBC ($10^6/mm^3$)	6.99 ± 0.52	7.28 ± 0.53 **(+4%)**	6.88 ± 0.36 **(-2%)**	7.50 ± 0.31 **(+7%)**	6.81 ± 0.28	7.10 ± 0.38 **(+4%)**	6.65 ± 0.22 **(-2%)**	7.03 ± 0.09 **(+3%)**
day 28								
Hb (g/dL)	16.4±0.9	16.5±0.7 **(+1%)**	15.6±0.5 **(-5%)**	14.9±0.7 **(-9%)***	16±0.8	15.8±1 **(-1%)**	14.9±0.7 **(-7%)**	14.7±0.8 **(-8%)**
Hct (%)	47.2±3.4	47.7±2.5 **(+1%)**	44.3±2.1 **(-6%)**	42.3±1.6 **(-10%)***	45.8±2.3	45.5±3 **(-1%)**	42.7±1.8 **(-7%)**	42.3±2.8 **(-8%)**

	M				F			
	Dose (ppm)							
	0	400	1200	3600/2400	0	400	1200	3600/2400
RBC (10^6/mm^3)	7.02 ± 0.40	7.13 ± 0.52 **(+2%)**	6.85 ± 0.23 **(-2%)**	6.64 ± 0.26 **(-5%)**	6.91 ± 0.35	6.99 ± 0.64 **(+1%)**	6.41 ± 0.33 **(-7%)**	6.42 ± 0.27 **(-7%)**
day 56								
Hb (g/dL)	16.1±1	16.8±0.7 **(+4%)**	15.1±0.8 **(-6%)**	14±1.1 **(-13%)***	16.4±0.8	16.6±1.2 **(+1%)**	15.3±0.7 **(-7%)**	13±1 **(-21%)***
Hct (%)	46.4±3.9	48±2.1 **(+3%)**	43.2±1.9 **(-7%)**	39.9±2.7 **(-14%)***	46.2±2.7	47.6±3.1 **(+3%)**	43±1.9 **(-7%)**	37.7±2.7 **(-18%)***
RBC (10^6/mm^3)	6.92±0.42	7.18±0.46 **(+4%)**	6.67±0.26 **(-4%)**	6.29±0.52 **(-9%)**	6.95±0.36	7.25±0.53 **(+4%)**	6.44±0.24 **(-7%)***	5.84±0.28 **(-16%)***
day 84								
Hb (g/dL)	16.3±1.5	17±1 **(+4%)**	15.5±0.9 **(-5%)**	14.9±0.3 **(-9%)**	15.9±0.8	16.9±1.1 **(+6%)**	15.2±0.4 **(-4%)**	14.1±1.9 **(-11%)**
Hct (%)	47.6±3.2	49.5±3.5 **(+4%)**	45.4±2.3 **(-5%)**	43.2±1 **(-9%)***	45.3±2.5	48.3±3.1 **(+7%)**	43.6±1.3 **(-4%)**	40.7±5.3 **(-10%)**
RBC (10^6/mm^3)	7.07 ± 0.44	7.36 ± 0.63 **(+4%)**	6.97 ± 0.30 **(-1%)**	6.75 ± 0.24 **(-5%)**	6.80 ± 0.40	7.31 ± 0.61 **(+8%)**	6.53 ± 0.21 **(-4%)**	6.25 ± 0.62 **(-8%)**

'*' - Denotes statistical significance at the p≤0.05 or ** p≤0.01 level. Data excerpted from pages 88 to 100

2. Clinical chemistry

Clinical chemistry findings at study day 28 were unremarkable for all groups (Table KIIA 5.3.3/01-7).

At study day 56, both sexes had marked increases in creatine kinase (CK)(approx. 10/4-fold in M/F at 1200 ppm, increasing to 11/16-fold at 3600/2400 ppm), aspartate aminotransferase (AST) (5/2-fold in M/F at 1200 ppm, increasing to 6/8-fold at 3600/2400 ppm), and alanine aminotransferase (ALT) (6/4-fold in M/F at 1200 ppm, increasing to 9/18-fold at 3600/2400 ppm).

There were also findings at 3600/2400 ppm at study day 56 in the form of decreased creatinine levels in serum (20/22% decreases in M/F, statistically significant at p<0.05 for F only), and a non-statistically significant increase of 17/59% (M/F) in serum cholesterol, and a statistically significant increase in glucose level in females (24%; p<0.05) when compared to controls. These findings at study day 56 had resolved by study day 84.

The only statistically significant clinical chemistry finding at day 84 was a minor 2% decrease (p≤0.05) in serum sodium in females at 3600/2400 ppm. Given the lack of dose and temporal relationship, this effect is considered incidental.

Overall, the study authors regarded that for both sexes at 1200 and 3600/2400 ppm, test substance related changes in clinical chemistry were increases in CK, AST, and ALT activity at study day 57, with resolution of these enzyme changes by study day 84. While the evaluator agrees with the study authors that the activity of these enzymes were comparable to controls in females at study day 84, ALT activity in males at this time point was still slightly elevated at 1200 and 3600/2400 ppm (Table KIIA 5.3.3/01-8). **Table KIIA 5.3.3/01-7: Clinical chemistry parameter findings at study day 56**

	M				F			
	Dose (ppm)							
	0	400	1200	3600/2400	0	400	1200	3600/2400
CK (U/L)	242±35	248±55 (2%)	2834±4554 (1071%)	2944±1986 (1117%)	282±71	175±56 (-38%)*	1538±1222 (445%)	4836±2895 (1615%)*
AST (U/L)	38±3	36±3 (-5%)	241±353 (534%)	266±127 (600%)	42±6	31±1 (-26%)*	130±75 (210%)*	370±211 (781%)*
ALT (U/L)	30±4	36±3 (20%)	221±284 (637%)	311±142 (937%)	28±3	27±5 (-4%)	151±83 (439%)*	552±205 (1871%)*
creatinine (mg/dL)	1±0	0.9±0 (-10%)	0.9±0.1 (-10%)	0.8±0.1 (-20%)*	0.9±0	0.9±0.1 (0%)	0.8±0.1 (-11%)	0.7±0.0 (-22%)*
chol (mg/dL)	144±25	140±25 (-3%)	160±33 (11%)	169±30 (17%)	132±24	182±59 (38%)	172±33 (30%)	210±34 (59%)

'*' - Denotes statistical significance at the p≤0.05 or ** p≤0.01 level. Data excerpted from pages 64 to 85.**Table KIIA 5.3.3/01-8: Clinical chemistry parameter findings at study day 84**

	M				F			
	Dose (ppm)							
	0	400	1200	3600/2400	0	400	1200	3600/2400
ALT (U/L)	33±5	35±7	46±11	51±17	25±2	31±4	32±6	31±7
		+6	+39	+55		+24	+28	+24

Not statistically significant in any group at the p≤0.05 level. Data excerpted from pages 64 to 85.

3. Urinalysis

Urinalyses parameters were unaffected by dosing.

At 3600/2400 ppm on study day 85, decreased urinary protein counts in were seen in males but there were already large variations in this parameter at pre-dosing on study day -6. Similarly, the decreases in urinary blood counts in females at day 85 were of similar extent to that seen on study day -6, and it is therefore regarded as unrelated to the test substance.

E. OPHTHALMOLOGY

Ophthalmological parameters were unaffected by dosing.

F. SACRIFICE AND PATHOLOGY

1. Organ weight

Statistically significant changes in organ weight parameters were confined to 1200 ppm and above, with 18% (p≤0.05) increases in relative kidney weight in males at 1200 ppm, increasing to a 31% increase (p≤0.05) at 3600/2400 ppm (Table KIIA 5.3.3/01-9). Relative kidney weights were also increased in a dose-related fashion in females, with a 16% increase over controls at 3600/2400 ppm (without attaining statistical significance). Absolute kidney weights in males were also increased relative to control, but without statistical significance or dose relationship. The study authors regarded the increases in relative kidney weights in males at 1200 ppm and relative kidney weights in both sexes at 3600/2400 ppm to be test substance related. The evaluator agrees with this conclusion.

Statistically significant (p≤0.05) increases in absolute (14/11% in M/F, statistically

significant in M only) and relative (28/24% in M/F, p≤0.05) liver weights were seen in both sexes at 3600/2400 ppm.

Additionally, increased absolute (67% increase, p≤0.05) and relative (88% increase, p≤0.05) weights of the prostate were seen at 3600/2400 ppm. Statistically significant (p≤0.05) increases of 55% in group mean relative (to bodyweight) prostate weights were also seen at 1200 ppm. While absolute prostate weight at 1200 ppm was elevated at similar levels (41% increase, p>0.05), no statistical significance was noted, and magnitude of the increase was comparable to increases at 400 ppm (34% absolute, 28% relative increase, p>0.05).

Based on the concordance of absolute and relative weight increases, increased liver weights in both sexes and prostate weights are considered to be test substance related at 3600/2400 ppm.

The study authors did not regard the statistically significant (p≤0.05) increases in prostate weights in males at 3600/2400 ppm to be test substance related, stating that large inter-individual differences in the development of sex-related organs is typical in young animals. The evaluator disagrees that there were any unusually large levels of variation in this parameter for test substance groups, as relative standard deviations for recorded values ranged from 14 to 18% of mean for test substance and 38% for controls. The evaluator also notes that a reasonable dose relationship was present for the finding in both absolute and relative (to bodyweight) weights, and that they were statistically significant (p≤0.05) in the 3600/2400 ppm group. As such, the evaluator therefore considers increased prostate weights at 3600/2400 ppm to be a test substance related finding. Based on the absence of statistical significance and similar extent to increases at 400 ppm, the evaluator considered the absolute weight increases of the prostate at 1200 ppm to be unrelated to the test substance.

There were no other dose dependent differences in absolute or relative organ weights between test substance and control groups.

Table KIIA 5.3.3/01-9: Organ weights at scheduled sacrifice on study days 91 or 92

	Male				Female			
Dose (ppm)	**0**	**400**	**1200**	**3600/2400**	**0**	**400**	**1200**	**3600/2400**
Liver Abs (g)	269.2± 21.687	260.35± 12.754	261± 35.654	307.1± 15.41	225.867± 27.027	201.47± 41.82	225.05± 22.405	251.575± 41.344
		(-3%)	**(-3%)**	**(+14%)***		**(-11%)**	**(0%)**	**(+11%)**
Liver Rel/BW	2.757± 0.264	2.533± 0.101	2.911± 0.339	3.525± 0.546	2.892± 0.256	2.621± 0.284	3.035± 0.088	3.596± 0.345
		(-8%)	**(+6%)**	**(+28%)***		**(-9%)**	**(+5%)**	**(+24%)***
Kidney Abs (g)	47.41± 1.13	55.218± 5.317	51.389± 8.049	55.076± 2.33	37.905± 2.8	33.612± 5.567	35.918± 2.261	37.925± 2.455
		(+16%)	**(+8%)**	**(+16%)**		**(-11%)**	**(-5%)**	**(+0%)**
Kidney Rel/BW	0.485± 0.021	0.539± 0.064	0.57± 0.048	0.633± 0.102	0.469± 0.01	0.442± 0.07	0.488± 0.057	0.545± 0.027
		(+11%)	**(+18%)***	**(+31%)***		**(-6%)**	**(+4%)**	**(+16%)**
Prostate (g)	7.375± 2.875	9.847± 1.547	10.41± 1.896	12.322± 1.75	NA			
		(+34%)	**(+41%)**	**(+67%)***				
Prostate Rel/BW	0.075± 0.029	0.096± 0.012	0.116± 0.019	0.141± 0.026				

	Male				Female			
Dose (ppm)	**0**	**400**	**1200**	**3600/2400**	**0**	**400**	**1200**	**3600/2400**
		(+28%)	**(+55%)***	**(+88%)***				

'*' - Denotes statistical significance at the p≤0.05 level. Data excerpted from 173 to 180, except for per cent change from controls which were calculated by the evaluator using Microsoft Excel.

2. Gross pathology and histopathology

Gross necropsy findings were unremarkable.

Accumulation of brown pigment to a minimal severity was seen in hepatic Kupffer cells of 2/4 females at 3600/2400 ppm, but was absent from all other groups. No further information was provided on the nature of these lesions (Table KIIA 5.3.3/01-10).

Skeletal muscle myofiber atrophy/degeneration was seen in 2/4 males (both minimal severity) and all four females (3 minimal, 1 slight severity) at 1200 ppm, and in 2/4 males at 3600/2400 ppm at slightly increased severity (1 minimal, 1 slight) and in all four females at 3600/2400 ppm but all of minimal severity. The study authors regarded these findings to be the cause of the marked increases for both sexes in the activities in serum of creatine kinase (CK) (approx. 10/4-fold in M/F, increasing to 11/16-fold at 3600/2400 ppm), aspartate aminotransferase (AST) (5/2-fold in M/F, increasing to 6/8-fold at 3600/2400 ppm), and alanine transaminase (ALT) (6/4-fold in M/F, increasing to 9/18-fold at 3600/2400 ppm) at study day 56. However, US EPA secondary reviewer considered the increased in clinical chemistry parameters (CK AST, & ALT) as the results of the skeletal myofiber degeneration.

Table KIIA 5.3.3/01-10: Histopathology findings in beagle dogs after 3 month treatment with BYI 02960

N=4	**Males**				**Females**			
	Dose (ppm)							
	0	**400**	**1200**	**3600/ 2400**	**0**	**400**	**1200**	**3600/ 2400**
Myofiber atrophy/degeneration: focal								
Minimal	**0**	**0**	2	1	**0**	**0**	3	4
Slight	**0**	**0**	**0**	1	**0**	**0**	1	-
Total	**0**	**0**	**2/4 (50%)**	**2/4 (50%)**	**0**	**0**	**4/4 (100%)**	**4/4 (100%)**
Liver: Accumulation of brown pigment in Kupffer cells, minimal severity								
Total	0	0	0	0	0	0	**0**	**2/4 (50%)**

Statistical analyses were not undertaken. Data excerpted from page 22–23 of the study report.

Overall, the study authors set a NOAEL in both sexes at 400 ppm (12 mg/kg bw/d), based on effects at 1200 ppm (~33/41 mg/kg bw/d for M/F) consisting of 9% decrease in terminal bodyweights in males relative to controls accounted for by decreased food consumption, increased CK, AST, and ALT at study week 8, and skeletal muscle myofiber atrophy/degeneration in males of minimal severity and females of minimal to slight severity.

The primary evaluator agrees with the study author that the NOAEL in both sexes was 400 ppm, based on adverse test substance related, gradual decreases on group mean bodyweights gains at 1200 ppm relative to controls at study week 3 onwards, with mean terminal bodyweight weights that were

9/7% lower than controls at study week 13 (statistically significant at $p<0.05$). US EPA secondary review does not believe the body weight changes approached the magnitude of being adverse. Additionally, skeletal muscle myofiber atrophy/degeneration was also seen in 2/4 males (both minimal severity) and all four females (3 minimal, 1 slight severity) at 1200 ppm.

III. CONCLUSION

Under the conditions of this study, BYI 02960 did not affect mortality, ophthalmology, or urinalysis parameters.

The no-observed-adverse-effect level (NOAEL) for both sexes was 400 ppm. The LOAEL was 1200 ppm, based skeletal muscle myofiber atrophy/degeneration was also seen in 2/4 males (both minimal severity) and all four females (3 minimal, 1 slight severity). In addition, associated increases in CK. AST, and ALT were also observed.

This study is classified as fully reliable (acceptable/guideline) and satisfies guideline requirements for a subchronic oral toxicity in dogs [OPPTS 870.3150; OECD 409].

Primary review: Australia
Secondary review: PMRA & US EPA

Report: Cada A (2012) A Subacute Dermal Toxicity Study in Rats with Technical Grade BYI 02960. Xenometrics, LLC. Stilwell, Kansas. Study No.**:** 11-S22-US. 5 June 2012. Unpublished. Study Sponsor: Bayer CropScience, Stilwell, Kansas. Bayer CropScience File No. M-432336-01-1. MRID 48844115. PMRA 2239546.

Guidelines: US EPA OPPTS 870.3200 (1998)
OECD Part 410 (1981)

Deviations: No deviations from test guidelines, except that there were eight instances where animals were found out of wrapping/dermal jackets at the time of unwrapping/washing; this deviation had no impact on the outcome of the study.

GLP: Yes. Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a 28-day dermal toxicity study (MRID 48844115), BYI 02960 (96.2%) was applied to the shaved, intact dorsal skin of male and female Wistar: Crl:WI(Han) rats (10/sex/dose). The test material was moistened with deionized water and applied onto approximately 58 cm^2 shave skin for 28 daily (consecutive) applications. The rats were exposed to the test material for a minimum of 6 hours per day at 0, 50, 150 and 500 mg/kg bw/day. Parameters evaluated included body weight, body weight gain, food consumption, clinical signs, clinical pathology, ophthalmology, organ weights, and gross and microscopic pathology.

Under the conditions of this study, no effects attributable to exposure to BYI02960 were observed in body weight, body weight gain, food consumption, clinical signs, clinical pathology, ophthalmology, or gross and microscopic pathology data.

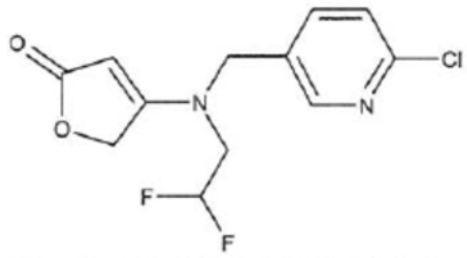
The absolute and relative liver weights at 500 mg/kg/day males were decreased by 6% and 9%, respectively, compared to control rats, with only the relative decrease obtaining statistical significance. There were no test material related clinical or gross or microscopic pathology changes to correlate this finding. **Consequently, based on the results of this study, the no-observed-adverse-effect-level (NOAEL) was 500 mg/kg bw/day (HDT) for both male and female rats.** The study report stated that the highest dose, 500 mg/kg bw, was selected after consideration of the amount of test material that could be reliably applied to the skin.

This study is considered to be fully reliable (acceptable/guideline) and to meet the data requirements for a 28-day dermal toxicity study (OPPTS 870.3200; OECD 410).

I. MATERIALS AND METHODS

A. MATERIALS

1 Test material: BYI 02960
4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one



Lot/Batch #:	2009-000239
Purity:	96.2%
Description:	Beige powder
CAS #	Not reported
Stability of test compound:	Stated to be stable for two years if stored at 20±10ºC.

2. Vehicle: deionized water

3 Test animals

Species:	Rat
Strain:	Wistar: Crl:WI(Han)
Age at dosing:	Approximately 8 weeks
Weight at dosing:	238.7–296.5 g for males; 157.7–193.4 g for females
Source:	Charles River Laboratories, Inc., Raleigh, NC
Acclimation period:	7 days
Diet:	Purina Mills Certified Rodent Diet 5002 in "meal" form, *ad libitum*.
Water:	Tap water, *ad libitum*
Housing:	Animals were housed singly in wire-bottomed, stainless steel cages with deotized cage board in the bedding trays.

4. Environmental conditions

Temperature:	18–26°C
Humidity:	30–70%
Air changes:	At least 17.79 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion

3 October to week of 31 October 2011

2. Animal assignment and treatment

The dosing regimen of 0, 50, 150, and 500 mg/kg bw/day was based on: (1) results from the acute dermal toxicity study of Gillissen (2009) where no observable toxicity was seen at 2000 mg/kg bw in rats; (2) the 28-day dietary study in rats by Blanck (2008; Study No. SA 07047) where decreased bodyweight gain and bodyweight was seen at 5000 ppm (385 mg/kg bw/day); and (3) the high dose of 500 mg/kg bw/day was stated to be selected after consideration of the amount of test material that can be reliably applied to the skin.

Four groups of 10 animals/sex/dose were administered dermal doses of BYI 02960 or vehicle control only daily (minimum 6 hours/day) for 28 days (Table 1). Animals were assigned to dose groups by computerised, stratified randomisation so that there were no statistically significant differences among group body weight means within a sex. Animal housing and husbandry were in accordance with the provisions of the *Guide to the Care and Use of Laboratory Animals* (USPHS-NIH Publication No. 86-23).

Table 1. Study design

Group No.	No./Sex/Group	Daily Dermal Dose mg/kg bw/d(A)
1	10	0 (control)
2	10	50
3	10	150
4	10	500

(A) – Dose level based on each animal's most recently determined bodyweight (Study Days 0, 7, 14, 21, and 28).

3. Dose preparation and analysis

For each individual animal on each day of application, the appropriate mass of test material was applied to 3 x 3 inch (7.62 x 7.62 cm, equal to 58.06 cm^2) square gauze pad pre-wetted with 2 mL (day 0) or 3 mL (days 1 to 28) deionized water and immediately applied to the shaved dorsal skin. Control animals received water vehicle alone. The test material was weighed out for each animal based on the most recently determined bodyweight. Based on this information, it can be concluded that the animals received the targeted dose of test material during the study.

4. Preparation and treatment of animal skin

On the day prior to the first application (Study Day -1), fur on the dorsal and lateral areas of the animal trunk (total surface area not reported) was closely clipped and checked for any abnormalities. The next day (Study Day 0), a 58.06 cm^2 piece of gauze containing pre-wetted test material (or containing vehicle only for controls) was applied to the shaved dorsal skin, and secured in place with a dermal dosing jacket. The test animals were dosed for at least 6 hours. Following the exposure period, dermal dosing jackets were removed

and the application site of each animal was gently wiped with water–dampened gauze as well as dry gauze to remove any test material. Shaving of the skin was undertaken on an as-needed basis over the 28 day duration (precise intervals not reported).

5. Statistics

The data were analyzed using applications provided by DATATOX. Statistical significance was determined at $p \leq 0.05$ for all tests with the exception of Bartlett's Test, in which a probability value of $p \leq 0.001$ was used. All tests were two-tailed, except for gross and histopathologic lesion evaluations that were one-tailed. Continuous data was analyzed by Bartlett's Test for homogeneity. If the data was homogeneous, an analysis of variance (ANOVA) was performed followed by Dunnett's t-Test on parameters showing a significant effect by ANOVA.

C. METHODS

1. Observations

Animals were observed at least twice daily (am and pm) for clinical signs of toxicity including mortality (observations were conducted once daily on weekends). Additionally, detailed clinical observations were made immediately prior to initial dosing and approximately weekly thereafter.

2. Body weights

All animals were weighed on Study Day 0 and on a weekly basis thereafter until Study Day 28 and prior to necropsy to obtain accurate relative organ weight calculations.

3. Food consumption and food efficiency

Food consumption was recorded at least weekly over the study duration. The study authors considered the food consumption values normalized for body weight (g food/kg bw/day) as food efficiency values.

4. Ophthalmological examinations

Ophthalmic examination was undertaken prior to dosing on Study Day 0 and within five days of scheduled sacrifice.

5. Clinical pathology (haematology, clinical chemistry, coagulation)

On Study Day 29, orbital sinus blood from all surviving animals was collected by capillary tube under isoflurane anaesthesia following an overnight fast (~500 µL for haematology, ~1000 µL for serum chemistry, and ~1800 µL for coagulation). Tubes collecting blood for haematology analysis contained EDTA, while those for coagulation contained sodium citrate. The following parameters were evaluated:

<u>Haematology and coagulation</u>

Red blood cell count/morphology	Red blood cell distribution width
Hemoglobin	Absolute reticulocyte count

Hematocrit	Platelet count
Mean corpuscular (cell) volume	Leukocytes (total & differential)
Mean corpuscular (cell) hemoglobin	Hemoglobin distribution width
Mean corpuscular(cell) hemoglobin concentration	
Activated partial thromboplastin time	Prothrombin time

Clinical chemistry parameters

alanine aminotransferase	albumin
A/G ratio	alkaline phosphatase
aspartate aminotransferase	bilirubin, total
blood urea nitrogen	calcium
chloride	cholesterol, total
creatinine	creatine phosphokinase
gamma-glutamyltranspeptidase	globulin
glucose	lactic dehydrogenase
phosphate	potassium
protein, total	sodium
triglycerides	uric acid

6. Sacrifice and pathology

At scheduled termination on Study Day 29, animals were sacrificed by CO_2 asphyxiation and all animals subjected to gross necropsy. Organs that were weighed and tissues that were preserved in 10% neutral buffered formalin are listed in Table 2. Organ weight to final body weight ratios were calculated for all dosing groups. Histological analysis was undertaken using haematoxylin and eosin staining and light microscopy examination. Only tissues from control and high dose groups were evaluated, with the exception of gross lesions, which were examined for any animal in which they arose.

Table 2. Organs/tissues collected for pathological examination

Tissue	Weighed	Collected/preserved	Histology
Adrenal gland (2)	X	X	X
Aorta		X	X
Bone marrow (sternum)		X	X
Brain (cerebrum, cerebellum, medulla/pons evaluated)	X	X	X
Cecum		X	X
Cervix		X	X
Clitoral gland		X	
Colon		X	X
Duodenum		X	X
Epididymis (2)	X	X	X
Esophagus		X	X
Exorbital/lacrimal gland		X	
Eye (2; preserved in Davidson's fixative)		X	X
Fallopian tube (oviduct) (2)		X	X
Femur (articular surface of distal end)		X	X
Gross lesions		X	X
Harderian gland (2; preserved in Davidson's fixative)		X	X
Heart	X	X	X
Ileum (include Peyer's patch)		X	X
Jejunum		X	X

Tissue	Weighed	Collected/preserved	Histology
Kidney (2)	X	X	X
Larynx		X	X
Liver	X	X	X
Lung with large bronchi		X	X
Lymph node (mandibular) (2)		X	X
Lymph node (mesenteric)		X	X
Mammary gland (both sexes)		X	X
Muscle (gastrocnemius)		X	X
Nasopharynx		X	X
Nasal structure		X	X
Optic nerve (2; preserved in Davidson's fixative)		X	X
Ovary (2)	X	X	X
Pancreas		X	X
Parathyroid gland		X	
Pituitary gland		X	X
Preputial gland		X	
Prostate		X	X
Rectum		X	X
Salivary gland [mandibular (2)]		X	X
Seminal vesicle (with coagulating gland) (2)	X	X	X
Sciatic nerve		X	X
Skin/subcutis (treated)		X	X
Skin (untreated)		X	X
Spinal cord (cervical, thoracic, and lumbar)		X	X
Spleen	X	X	X
Stomach		X	X
Testis (2; preserved in modified Davidson's fixative)	X	X	X
Thymus	X	X	X
Thyroid with parathyroid (2)		X	X
Tongue		X	X
Trachea		X	X
Urinary bladder		X	X
Uterus	X	X	X
Vagina		X	
Zymbal's gland		X	

Data excerpted from pages 20 and 21 of the study report.

Skin at the dosing site was included in the list of tissues histologically examined. Although the clitoral gland, exorbital/lacrimal gland, preputial gland, vagina, zymbal gland, and parathyroid gland tissue were collected and preserved, they were not analysed.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

No clinical signs of systemic toxicity were observed.

2. Dermal irritation

There were no reports of irritation during in-life observation, and no abnormalities reported for test material exposed skin at either necropsy or at

histological examination.

3. Mortality

There were no unscheduled mortalities.

B. BODY WEIGHT AND BODY WEIGHT GAIN

There were no test material related effects on body weight or body weight gain in any group (Table 3).

Table 3. Mean body weights and body weight gains

Study day	Body weight (g)							
	Males				Females			
	Dose (mg/kg bw/day)							
	0	50	150	500	0	50	150	500
0	264±13	264±17	265±14	265±18	178±9	177±9	176±10	176±11
7	280±17	282±22	280±17	285±23	192±9	187±7	193±15	189±13
14	292±16	299±28	293±23	302±27	201±10	197±9	202±15	197±12
21	307±18	315±30	307±25	319±30	209±10	205±8	211±15	203±10
28	323±19	331±32	324±28	336±32	218±12	215±9	218±17	215±13
Overall BWG	59	67	59	71	40	38	40	39

Data excerpted from pages 22, 32, and 33 of the study report.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

A decrease in relative food consumption (g food per kg bw/d) with poor dose dependency was seen in all test material group females, being apparent over Study Week 1 and lasting up to Study Week 4 excluding females administered 150 mg/kg bw/day in week 3 (Table 4).

The study author considered that the effects on food consumption in females were not treatment related, as they were not consistently affected in a dose-related fashion, were not found in males, and did not impact clinical observations or body weights at any time point. The study author's reasoning and conclusions were considered appropriate.

Table 4. Mean relative food consumption in females

Food consumption (g food per kg bw/day)				
	Dose (mg/kg bw/day)			
Study day	0	50	150	500
7	96.47±9.34	89.18±6.00 (-7.6%)	94.33±7.68 (-2.2%)	86.31±10.26* (-10.5%)
14	100.46±9.21	93.05±6.30 (-7.4%)	94.31±7.24 (-6.1%)	92.87±7.98 (-7.6%)
21	94.89±10.81	91.02±5.75 (-4.1%)	95.79±7.11 (+0.9%)	93.00±5.42 (-2%)
28	91±10.4	84.00±5.26 (-7.7%)	84.20±6.37 (-7.5%)	85.81±4.25 (-5.7%)

* Statistically significant from control at $p<0.05$
Data excerpted from page 35 of the study report.

D. OPHTHALMOLOGICAL EXAMINATIONS

No ophthalmological changes were noted in male or female rats at any dose level.

E. CLINICAL PATHOLOGY

1. Haematology

Relative to controls, statistically significant ($p\leq0.05$) decreases in erythrocyte count (7.1%) and haematocrit (5.3%) were seen in males at 150 mg/kg bw/d only, and a statistically significant decrease in prothrombin time (5.7%) was seen in females at 500 mg/kg bw/d (Table 5). The changes in erythrocyte counts and haematocrit in one sex were not considered test material related, based on the absence of dose dependency.

The findings in prothrombin time in females from all dose groups including concurrent controls were outside the historical control range (13.2 – 16.2 seconds, mean 14.8 seconds) in 10 to 17 week-old female Wistar (Hannover strain) rats from previous Bayer CropScience LP laboratory, making interpretation of the findings more difficult. However, the observed findings in one sex were considered spurious given that the decrease was associated with no other statistically significant treatment related changes in other haematology parameters and imparted no toxicological adverse consequences and that clotting time was not affected in any other study conducted with BYI 02960.

Table 5. Selected haematology parameters

Parameter	Dose (mg/kg bw/day)			
	0	**50**	**150**	**500**
	Males			
Erythrocyte count ($10^{\wedge 6}/mm^3$)	9.24±0.48	8.90±0.30 (-3.7%)	**8.58±0.41*** **(-7.1%)**	9.11±0.26 (-1.4%)
Haematocrit (%)	47.6±1.6	47.4±1.2 (-0.4%)	**45.1±2.1*** **(-5.3%)**	47.7±1.0 (+0.2%)
	Females			
Prothrombin time (seconds)	17.6±0.6	17.5±0.6 (-0.6%)	17.0±0.6 (-3.4%)	**16.6±0.6*** **(-5.7%)**

* Statistically significant from control at $p<0.05$

Data excerpted from pages 131 to 134 of the study report.

2. Clinical chemistry

Statistically significant changes in clinical chemistry parameters were confined to females at doses of 150 mg/kg bw/d and above, with increases of 1.4% - 2.1% in sodium ($p\leq0.05$), and decreases of 9.1% in potassium ($p\leq0.05$), and 22% in uric acid ($p\leq0.05$). Potassium and uric acid findings in one sex did not demonstrate dose dependency, and all these clinical chemistry findings were within the submitted historical control range (and within two standard deviations of the historical control mean) in 10 to 17 week-old female Wistar (Hannover strain) rats from previous Bayer CropScience LP laboratory studies (Table 6). Consequently, these observed clinical chemistry changes in one sex were not considered to be related to treatment with BYI 02960.

Additionally, at 500 mg/kg bw/d in females, triglyceride levels were statistically significantly increased by 23% ($p\leq0.05$) relative to controls, with poor dose dependency seen and which were within the historical control range. A slight, non-statistically significant increase in triglycerides (27%) was also noted in males from the 500 mg/kg bw/d dose group when compared to controls; values for males were also within the historical control range. Overall, the changes in triglycerides are considered to be spurious and unrelated to treatment.

Uric acid levels were not examined in males, though in this instance this deviation was not considered to not impact on the reliability of the study findings and conclusions.

Table 6. Selected clinical chemistry parameter in female rats

Parameter	Dose (mg/kg bw/day)				Historical control mean (and range) [N=50[A]]
	0	50	150	500	
Males					
Triglycerides (mg/dL)	48±17	46±19	46±13	**61±19 (+27%)**	**50 (17-154)**
Females					
Na (mmol/L)	140±1	141±1 (+0.7%)	**142±1* (+1.4%)**	**143±2* (+2.1%)**	**142 (137 – 146)**
K (mmol/L)	4.4±0.3	4.3±0.3 -2.3%	**4.0±0.2* (-9.1%)**	**4.0±0.2* (-9.1%)**	**4.6 (3.7 – 5.7)**
Uric A (mg/dL)	0.9±0.1	0.8±0.2 -11.1%	**0.7±0.1* (-22.2%)**	**0.7±0.2* (-22.2%)**	**1.0 (0.3 – 1.7)**
Trig (mg/dL)	30±4	36±7 +20%	35±6 +16.7%	**37±4* (+23.3%)**	**30 (15 – 53)**

[A]: N = 57 for triglyceride historical control data in males, and 43 for uric acid historical control data.
* Statistically significant from control at $p<0.05$
Data excerpted from pages 141, 144, 310 and 311 of the study report.

F. SACRIFICE AND PATHOLOGY

1. Organ weight

In males at 500 mg/kg bw/day, absolute liver weights were decreased by 6% (not statistically significant) and relative liver weights were decreased by 9% ($p\leq0.05$) relative to controls. In the absence of accompanying changes in the liver observed at gross necropsy or histopathological examination, the observed findings in one sex were not considered to be toxicologically significant.

Statistically significant ($p\leq0.05$) decreases in absolute (16%) and relative (15%) ovary weights in females at 50 mg/kg bw/day only were considered to be unrelated to the test material.

Results for all other absolute and relative weights of organs were unremarkable.

2. Gross pathology and histopathology

No test material related gross or microscopic lesions were found in any dosed groups relative to the controls.

III. CONCLUSION

The study author considered the no-observed-adverse-effect-level (NOAEL) in both sexes to be 500 mg/kg/day for both male and female rats. The evaluator agrees with the identified NOAEL in both sexes and interpretation of the study findings.

In this 28-day dermal toxicity study in Wistar rats with up to 500 mg/kg bw/day BYI 02960, there was no mortality, no test material related haematology or clinical chemistry change, no gross pathology change and no test material related terminal body weight change or test material related histopathology change in either sex at any dose level tested.

After 28 days of repeated dermal exposure to the test material, the toxicological response of the rat was limited to mild decreases in absolute and relative liver weights in males at 500 mg/kg bw/day with no clinical pathology or micropathology correlates. Based on the results of this study, the NOAEL in this study was 500 mg/kg/day for both male and female rats.

A lowest-observed-adverse-effect-level (LOAEL) was not identified in this study, but noting that a rationale was provided for the top dose selection this was not considered to affect the reliability of the study in this instance.

This study is considered to be fully reliable (acceptable/guideline) and to meet the data requirements for a 28-day dermal toxicity study (OPPTS 870.3200; OECD 410).

Primary review: Australia
Secondary review: PMRA & US EPA

Report: Langrand-Lerche, C (2010) BYI 02960: Developmental toxicity study in the rat by gavage. Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France. Report No. SA 08347. February 22, 2010. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N°TXRVP025. Bayer CropScience File No. M-363938-01-2. MRID 48844116. PMRA 2239547. Unpublished.

Guidelines: OPPTS 870.3700 (1998)
OECD 414 (2001)
EC Directive 2004/73 Method B.31 (2004)
MAFF 12 Nousan 8147 (2000)

Deviations: None

GLP: Yes Signed statements of GLP, Quality Assurance, and Data Confidentiality were presented in the report.

Executive summary:

In a developmental toxicity study (MRID 48844116), groups of mated Crl:CD®(SD) female rats (23/dose group) received BYI 02960 (96.2% purity) in 0.5% aqueous methylcellulose 400 vehicle at doses of 0, 15, 50, or 150 mg/kg bw/day (10 mL/kg bw dosing volume) on gestation days (GD) 6 to 20. For dams, parameters evaluated were: bodyweight, bodyweight gain, food consumption, survival, clinical signs, reproductive outcomes, and gross pathology. Fetal parameters evaluated were: fetal bodyweight, incidences of dead fetuses and/or fetal resorptions, and incidences of external, visceral, head, and skeletal malformations and variations.

For dams, there were no test substance-related effects on survival, reproductive outcomes, or gross necropsy findings. The mean number of corpora lutea, implantation sites, resorptions, live fetuses, fetal weight and sex ratio were comparable across all groups and controls. An increased incidence of salivation (20/23) in 150 mg/kg bw/day animals was noted and considered a treatment-related effect. Additionally, a lower gestational bodyweight gain in females was noted as a treatment-related effect, with a 16% lower corrected bodyweight gain at 150 mg/kg bw/day compared with controls, and statistically significant decreased food consumption in the same dose group over GD 6 to 12 compared with controls. No adverse maternal effects were observed in the 50 and 15 mg/kg bw/day groups.

In fetuses, there were no test substance-related effects on the incidence of malformations or external variations. However, at 150 mg/kg bw/day, the incidence of the variations “parietal (unilateral/bilateral): incomplete ossification” and “hyoid centrum: incomplete ossification” were higher than in the control group and were outside the in-house historical control at both litter and fetal levels. These findings were indicative of a slightly delayed fetal development and considered to be test substance related. No adverse developmental effects were found in 50 and 15 mg/kg bw/day fetuses.

The maternal NOAEL was 50 mg/kg bw/day, and maternal LOAEL was 150 mg/kg bw/day, based on salivation, lower gestational bodyweight gain and decreased food consumption during the treatment period.

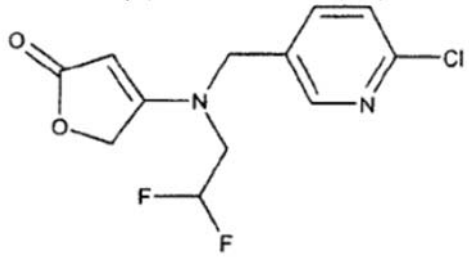
The developmental NOAEL was 50 mg/kg bw/day; developmental LOAEL was 150 mg/kg bw/day, based on increased incidences of incomplete parietal and hyoid centrum skull bone ossification indicative of delayed fetal development.

This study is fully reliable (acceptable/guideline) and satisfies the requirements for a developmental toxicity study in rats **(**OPPTS 870.3700; OECD 414; Directive 87/302/EEC Part B; MAFF 12 Nousan 8147).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one



Batch #:	2009-000239
Purity:	96.2% (method No. 2960C01; Jan 16 2009)
Description:	Beige powder
CAS #:	951659-40-8
Stability of test compound:	Stored in an air-tight, light-resistant container at room temperature. Stated to be stable for two years at 25±5ºC. The study authors cited Report No. SA 07025 (Amir Tamassee, 2007), which reported stability of test compound for 28 days in aqueous methylcellulose at 1 and 250 g/L concentrations at room temperature. In this present study, test compound homogeneity was analysed, with prepared samples reporting 93 to 101% of nominal concentrations.

2. Vehicle and/or positive control: 0.5% aqueous methylcellulose 400
3. Test animals

Species:	Rat (female)
Strain:	CRL: CD®(SD)
Age at dosing:	Approximately 12 to 16 weeks old
Weight at dosing:	Approximately 225 to 289 g (GD 0)
Source:	Charles River Laboratories, St Germain-sur-l'Arbresle, France
Acclimation period:	At least 12 days prior to mating (GD 0)
Diet:	A04C-10 pelleted rodent diet from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*.
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Individually in stainless steel, wire-mesh cages.

4. Environmental conditions

Temperature:	20–24°C
Humidity:	40–70%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion

17 February 2009 to 11 December 2009

2. Animal assignment and treatment

A total of 120 adult nulliparous female rats were mated on a one-to-one basis with stock males from the same animal supplier. On the morning following pairing, females with sperm plugs or spermatozoa as evidenced by vaginal smear were presumed to be pregnant and randomised to the study as Gestation Day 0 (GD 0). A total of 23 presumed pregnant dams per group were randomised to one of four dosing groups. Assignment to groups was based on similarity in bodyweight means across groups, and where possible dams pregnant to the same male were placed into different dosing groups.

The dosing regimen used in the current study was based on the results of a preliminary dose finding study (Kennel 2007; SA 06249) where eight dams received doses up to 350 mg/kg bw/day from GD 6 to 20 experienced increased clinical signs consisting of salivation, soiled mouth and anogenital region, vaginal discharge, decreased bodyweight gain associated with decreased food consumption, decreased bodyweight corrected for gravid uterus weight, and increased liver weight at doses ≥200 mg/kg bw/day. The percentage of pre- and post-implantation losses were increased at 350 mg/kg bw/day and the number of live fetuses per litter and mean fetal weight were decreased at ≥200 mg/kg bw/day. A few variations were noted at external and skeletal observation at 350 mg/kg bw/day. Slight decreases in food consumption and maternal bodyweight fetusesfetalwere seen at 100 mg/kg bw/day. The study authors regarded the NOEL fetusesto be 50 mg/kg bw/day in the preliminary study.

Therefore, based on preliminary results, doses of 0, 15, 50, or 150 mg/kg bw/day BYI 02960 (w/v) in 0.5% aqueous methylcellulose 400 (10 mL/kg bw dosing volume) were selected for the main study (Table KIIA 5.6.10/01-1). Test substance was administered to dams on GD 6 to 20, with the date where females presented with sperm-positive observations denoted as GD 0. Dosing volumes administered to animals were based on the most recent body weight of each animal. Negative controls received 0.5% aqueous methylcellulose only. On GD 21, all surviving dams were sacrificed by CO_2 asphyxiation.

Table KIIA 5.6.10/01-1 Study design

Group no. [N=23/group]	Dosing duration (GD)	Dose (mg/kg bw/day)	Test formulation concentration (g/L)[b]	Animal ID No.'s
1	GD 6 to 20	0	0	TR1F0111 to 0133
2		15	1.5	TR2F0134 to 0156
3		50	5	TR3F0157 to 0179
4		150	15	TR4F0180 to 0202

[b] dosing volume 10 ml/kg bw. Data excerpted from page 17 of the study report.

3. Dosing suspensions, preparation, and analysis

Suspensions of test substance in 0.5% aqueous methylcellulose were prepared twice for each dosing level during the study, with batches being denoted F1 and F2. The test substance concentrations in the formulation were 1.5, 5, and 15 g/L for the 15, 50, and 150 mg/kg bw/day nominal dosing concentrations, respectively. When not in use, the suspensions were

stored at 5±3°C, and prior to use, suspensions were mixed continuously before and during dosing with an electromagnetic stirrer.

Homogeneity analysis using HPLC was undertaken on the top, middle, and bottom layer of the first batch of the low and high dosing formulations (Table KIIA 5.6.10/01-2). Additionally, concentration analysis using HPLC was undertaken on surface layer samples of the 5 g/L F1 formulation, and in all dosing concentrations of the F2 batches. The achieved mean concentrations in the dosing suspension were found to be 96% (15 g/L F1 dosing formulation) to 101% (1.5 g/L F1 dosing formulation) of nominal concentrations, and therefore met the laboratory's in-house criteria of 90 to 110% of nominal concentrations. The minimum concentration reported in analyses were 93% of nominal concentration, obtained from the middle layer of the 15 g/L F1 formulation (noting an overall mean of 96% nominal concentration for the top, middle and bottom layers).

Table KIIA 5.6.10/01-2: Homogeneity/achieved concentration analysis.

Batch ID	Date of analysis (date of formulation)	Layer obtained from batch (H, M, B)	Achieved concentration (g/L)	% of nominal (and per cent St Dev)
0 g/L				
F1	4 Mar, **2009**	H	<VL	-
F2	17 Mar, **2009**	H	<VL	-
1.5 g/L				
F1	4 Mar, **2009**	H	1.51	101
		M	1.51	101
		B	1.51	101
		H to B Mean	**1.510±0.0**	**101±0.0**
F2	17 Mar, **2009**	H	1.47	**98**
5 g/L				
F1	4 Mar, **2009**	H	4.98	**100**
F2	17 Mar, **2009**	H	4.97	**99**
15 g/L				
F1	4 Mar, **2009**	H	14.5	97
		M	13.9	93
		B	14.8	99
		H to B Mean	**14.40±0.46**	**96±3.1**
F2	17 Mar, **2009**	H	**15.0**	**100**

'VL' – denotes verifiable limit, equal to 0.1 g/L. 'H' – denotes surface layer, 'M' denotes middle, and 'B' denotes bottom layer.

Stability analysis was not undertaken, with the study authors citing Report No. SA 07025 (Amir Tahmasseb, 2007) where the test substance was stated to be stable in aqueous methylcellulose at 1 and 250 g/L concentrations over 28 days at room temperature.

4. Statistics

The statistical methods employed are presented in Table KIIA 5.6.10/01-3, and proceeded using the program TERATEST Phase 1, V. 12, or SAS programs (Version 8.2). Statistical analyses were performed separately for all pregnant females and for all pregnant females with live fetuses. Group means were compared at the $p \leq 0.05$ and $p \leq 0.01$ levels. Body weight data measured on different days throughout gestation were not statistically analysed, with only descriptive statistics being presented.

Fetal mortality was analysed both using the foetus as the statistical unit and using the litter as the statistical unit.

Table KIIA 5.6.10/01-3. Statistics used in data analyses

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant (p>0.05)	**If preliminary test is significant (p≤0.05)**
Bodyweight change (absolute and corrected for gravid uterus weights). No. of corpora lutea, implantation sites, and resorptions (early & late).	**Bartlett Test**	Analysis of variance followed by 2-sided **Dunnett's test if significant**	**Kruskal-Wallis test** followed by 2-sided **Dunn's test if significant**
Mean food consumption Liver weights Pre- and post-implantation loss	**Bartlett Test**	Analysis of variance followed by 2-sided **Dunnett's test if significant**	**Bartlett Test** on log transformed[A] data**,** if significant then **Kruskal-Wallis test** followed by 2-sided **Dunn's test if significant;** Otherwise **ANOVA, followed by Dunnett tests** on transformed data if significant
Fetal bodyweight	**Levene Test**	Analysis of variance followed by 2-sided Dunnett's test if significant	Levene Test on log transformed data; if significant then Kruskal-Wallis test followed by 2-sided Dunn's test if significant; Otherwise ANOVA, followed by Dunnett test on log transformed data if significant
Sex ratio	**Chi-square test** fetal		
Fetal mortality	**2-sided Fisher Exact test** fetal		
Skeletal observations	**Fisher Exact test**		

[A] – except for implantation loss, which was transformed using arcsine root transformation.

C. METHOD

1. Observations

From GD 0 to GD 21, dams were observed daily for clinical signs and twice daily for mortality and morbidity (once daily on weekends/public holidays), and any abnormal findings were recorded.

2. Body weights

All dams were weighed on GD's 0, 6, 8, 10, 12, 14, 16, 18 and 21.

3. Food consumption

Food consumption for dams were measured at GD 1, 6, 8, 10, 12, 14, 16, 18 and 21. Mean daily consumption was calculated from measured values. Food spillage was also noted where appropriate.

4. Sacrifice and pathology (maternal animals)

At termination (gestation Day 21), animals were sacrificed by carbon dioxide inhalation. Gross necropsy was performed on all animals, and included examination of viscera and recording of gravid uterus weights. The liver of each pregnant dam was weighed, and a portion retained and fixed in 10% neutral buffered formalin for potential histopathological examination (livers were not examined). The reproductive tract of each pregnant dam was removed and dissected to permit examination of the contents. Uterine horn(s) without visible implantations were immersed in a 10% solution of ammonium sulfide to assist in identification of implantation sites.

5. Reproductive outcomes

The following reproductive parameters were evaluated: pregnancy rate, premature delivery rate, total resorption rate, mean number of implantations, live fetuses, dead fetuses, corpora lutea counts, and pup sex ratio. Intra-uterine mortality was classified as either an early or late resorption based on the criteria of Gleich & Frohberg (1977). Specifically, early resorption occurred when macroscopic discrimination between fetal residues and placental material was not possible, and late resorption occurred when distinct macroscopic discrimination between fetal and placental tissue was possible.

6. Evaluation of fetuses

Parameters evaluated in fetuses were mean live and dead bodyweight, and incidences of external, visceral, head, and skeletal malformations and variations. All live fetuses were sacrificed by subcutaneous injection of sodium pentobarbital, and were counted, weighed, and externally examined. Approximately half from each litter were fixed in Bouin's solution and examined by dissection for soft tissue findings. The remaining half were eviscerated, skinned, fixed in absolute ethanol and stained according to a modification of the Tyl and Marr technique (1977) to allow for examination of bone and cartilage morphology. Dead fetuses were defined as dead conceptuses showing distinct digits on fore- and hind-paws.

II. RESULTS AND DISCUSSION

A. MATERNAL EFFECTS

1. Clinical signs of toxicity

Clinical signs were confined to dams at 150 mg/kg bw/day, where increased incidence of salivation was observed in 20/23 animals on at least one occasion each over GD 11 to 21, and one female at this dose was found to have soiling around the mouth on GD 21. Neither of these findings were seen in any other test substance group or controls.

2. Mortality

There was no unscheduled mortality.

3. Body weight, body weight gain, and food consumption

Mean bodyweights in the 150 mg/kg bw/day dams were within 4% of controls throughout the study period (Appendix A). Relative to body weights gains in controls, GD 6 to 21 body weight gains corrected for gravid uterus weights were 16% lower in dams at 150 mg/kg bw/day. This difference was largely due to a total bodyweight loss of 5.7 g ($p \leq 0.01$) over GD 6 to 8 (first three days of dosing) in these dams compared to a bodyweight gain of 5.9 g

in controls. Dams at 50 mg/kg bw/day also experienced a 49% lower mean bodyweight gain than control dams over GD 6 to 8, but the finding was without statistical significance (*i.e.*, p was >0.05) and overall bodyweight gain was unaffected at this dose (Table **KIIA 5.6.10/01-4**).

Bodyweights and bodyweight gains were unaffected by treatment at 15 mg/kg bw/day.

Bodyweight loss at 150 mg/kg bw/day over GD 6 to 8, and lower bodyweight gains at 50 mg/kg bw/day over GD 6 to 8 and at 150 mg/kg bw/day over GD 6 to 10 were accompanied by decreases in food consumption at 50 mg/kg bw/day (8%) and at 150 mg/kg bw/day (9–27%) (Table KIIA 5.6.10/01-5). Food consumption from GD 12 to 21 was unaffected by treatment at all doses.

Table KIIA 5.6.10/01-4: Maternal bodyweight gain (g)

	Dose (mg/kg bw/day)				HC
	0	15	50	150	
No. pregnant dams	**22**	**22**	**22**	**23**	
GD 0 to 6 (pre-dose)	29.4±8.08	25.3±7.50 (-14%)	27.6±5.53 (-6%)	28.5±5.44 (-3%)	28.05 - 39.25
GD 6 to 8:	5.9±3.82	5.9±2.78 (0%)	3.0±3.75 (-49%)	-5.7±5.18** (-197%)	4.05 - 8.48
GD 8 to 10:	9.1±1.96	7.4±2.28* (-19%)	8.5±2.56 (-7%)	6.9±3.48* (-24%)	7.78 - 11.30
GD 10 to 14:	16.8±3.84	17.1±7.81 (+2%)	16.1±5.22 (-4%)	19.5±4.09 (+16%)	17.17 - 22.33
GD 14 to 18:	41.0±8.88	43.8±7.06 (+7%)	44.9±6.48 (+10%)	41.7±7.64 (+2%)	41.48 - 48.21
GD 18 to 21:	43.6±9.61	44.9±17.09 (+3%)	47.5±7.59 (+9%)	45.7±8.89 (+5%)	47.09 - 58.21
GD 6 to 21	116.4±18.77	119.1±25.52 (+2%)	120.1±16.94 (+3%)	108.2±16.75 (-7%)	122.55 - 143.29
Corrected BW gain	44.6±16.80	40.9±18.89 (-8%)	44.0±12.81 (-1%)	37.3±8.89 (-16%)	49.56 - 75.43

'Corrected BW' – denotes bodyweight minus gravid uterus weight. 'HC' – denotes historical control data. * Denotes statistically significantly different from control at the *$p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from page 29 of the study report, with the exception of per cent change from control which was calculated by the evaluator using Microsoft Excel.

Table KIIA 5.6.10/01-5: Mean food consumption (g/d) in pregnant dams

	Dose (mg/kg bw/day)			
	0	**15**	**50**	**150**
No. of pregnant dams	**22**	**22**	**22**	**23**
GD 1 to 6:	23.9±2.77	23.0±2.56 (-4%)	23±2.01 (-4%)	24.0±1.72 (0%)
GD 6 to 8:	23.8±3.13	22.6±2.29 (-5%)	21.8±1.92* (-8%)	17.3±2.98** (-27%)
GD 8 to 10:	23.2±3.35	22.9±3.08 (-1%)	23.6±3.66 (+2%)	19.3±2.42** (-17%)
GD 10 to 12:	24.7±2.67	23.6±3.01 (-4%)	23.9±1.52 (-3%)	22.6±1.69* (-9%)
GD 12 to 14:	25.0 ± 1.69	24.3 ± 3.74 (-3%)	24.6 ± 1.93 (-2%)	24.0 ± 2.00 (-4%)
GD 14 to 16	25.4 ± 3.11	24.5 ± 3.37 (-4%)	26.1 ± 2.78 (+3%)	25.4 ± 2.33 (0%)
GD 16 to 18	26.5 ± 2.05	26.6 ± 4.40 (0%)	27.7 ± 2.07 (+5%)	27.8 ± 3.06 (+5%)
GD 18 to 21	24.6 ± 2.43	23.8 ± 5.86 (-3%)	25.5 ± 2.21 (+4%)	26.2 ± 2.67 (+7%)

* Denotes statistically significantly different from control at the *p≤0.05 or ** p≤0.01 level. Data excerpted from page 53–54 of the study report, with the exception of per cent change from control which was calculated by the evaluator using Microsoft Excel.

4. Reproductive outcomes

   There were no test substance-related effects on reproductive parameters or litter data endpoints, including number of live fetuses per litter, number of implantation sites per dam, percentages of pre- and post-implantation losses, early or late resorptions, fetal death status, or sex ratio (Table KIIA 5.6.10/01-6).

5. Gross pathology

   Necropsy findings in dams were unremarkable.

B. FETAL EFFECTS

No treatment-related effects on fetal body weight were noted (Table KIIA 5.6.10/01-6): the minor decrease (-2–3%) in fetal body weight at 150 mg/kg b/wd was not considered toxicologically adverse. There were no test substance-related effects on the incidence of malformations or external variations, with sporadic malformations at 15 mg/kg bw/day considered incidental. However, at 150 mg/kg bw/day, the incidence of the variations “parietal (unilateral/bilateral): incomplete ossification” and “hyoid centrum: incomplete ossification” were higher than in the control group and were outside the in-house HC at both litter and individual fetal levels (Table KIIA 5.6.10/01-7). These findings were indicative of a slightly delayed fetal development and are considered to be test substance related.

Table KIIA 5.6.10/01-6: Reproductive parameters

	Dose (mg/kg bw/day)				HC
	0	15	50	150	
Maternal data					
No. Animals assigned	23	23	23	23	NA
No. Animals pregnant	22	22	22	23	NA
Pregnancy rate, %	96	96	96	100	NA
No. Animals non-pregnant	1	1	1	0	NA
Maternal wastage **No. died (total)** **No. died pregnant** **No. died non-pregnant** **No. premature delivery**	 0 0 0 0	 0 0 0 0	 0 0 0 0	 0 0 0 0	 NA NA NA NA
Uterine data					
Total No. corpora lutea[c]	370	380	361	377	NA
Corpora lutea / dam	16.8±2.0	17.3±2.4	16.4±1.7	16.4±2.2	15.74 to 17.83
Total No. implantations[c]	329	349	334	345	NA
Implantations / dam	15.0±2.8	15.9±1.8	15.2±1.8	15.0±2.4	14.38 to 16.04
Total No. litters[c]	22	22	22	23	N/A
Total No. live fetuses[c]	304	319	313	314	NA
Live fetuses / dam[c]	13.8±3.0	14.5±2.2	14.2±2.3	13.7±2.5	13.52 to 15.04
Total No. dead fetuses	0	0	0	0	NA
Dead fetuses / dam[c], %	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0 to 0.30
Early resorptions[c] **Late resorptions[c]** **Early resorptions / dam** **Late resorptions / dam** **Litters with total resorptions[c]**	21 4 1.0±1.1 0.2±0.4 0	28 2 1.3±1.3 0.1±0.3 0	21 0 1.0±1.3 0.0±0.0 0	30 1 1.3±1.3 0.0±0.2 0	NA NA 0.474 to 2.042 0.0 to 0.217 NA
Mean fetal weight, M & F (g) **Mean fetal weight, M (g)** **Mean fetal weight, F (g)**	5.41±0.28 5.54±0.28 5.26±0.29	5.27±0.60 5.36±0.60 5.18±0.59	5.35±0.24 5.46±0.29 5.23±0.24	5.27±0.25 5.40±0.29 5.14±0.25	5.30 to 5.57 5.45 to 5.70 5.16 to 5.42
Sex ratio, % male	51.3±12.3	52.9±14.1	46.7±15.4	51.7±10.3	46.2 to 53.5
Preimplantation loss per litter, %	10.5±16.2	7.4±9.2	7.2±9.3	8.3±10.6	3.32 to 13.21
Postimplantation loss per litter, %	7.9±10.3	8.6±8.4	6.5±9.3	9.0±8.3	3.95 to 13.15

'c' – denotes that statistical analysis was not conducted for that parameter. No findings were statistically significant ($p \leq 0.05$). 'HC' – denotes historical control. Data derived from page 30 of the study report.

There were no other treatment-related fetal skeletal variations observed at any dose level. The incidence of the variations "7th cervical centrum: unossified" and "5th sternebra: incomplete ossification" were higher at 150 mg/kg/day than in the control group, but were not statistically significant. In the absence of a clear dose dependency, and noting all values were well within the in-house HCD at both the litter and fetal level, these findings were considered to be unrelated to the test substance. Additionally, the incidence of "13th costal cartilage (unilateral): short" was higher at 150 mg/kg/day than in the control group and was outside the in-house HCD at both litter and fetal levels. However, as the observation was noted unilaterally only and was of a low incidence (2 cases in 2 litters), it was not considered to be test substance-related.

All other variations were considered sporadic in nature.

Table KIIA 5.6.10/01-7: Selected findings from skeletal examination

Observation	Dose (mg/kg bw/day)				HC range (%)	Dose (mg/kg bw/day)				HC range (%)
	0	15	50	150		0	15	50	150	
	No. litters examined					No. fetuses/heads examined				
	22	22	22	23		158	165	161	162	
	No. of litters affected (% affected)					No. of fetuses affected (% affected)				
Variations # Parietal (uni/bi) : incomplete ossification.	0 (0.0)	2 (9.1)	0 (0.0)	4 (17.4)	**0.0 to 9.1**	0 (0.0)	6 (3.6)*	0 (0.0)	9 (5.6)**	**0.0 to 1.3**
# Hyoid centrum: incomplete ossification.	0 (0.0)	2 (9.1)	1 (4.5)	4 (17.4)	**0.0 to 12.5**	0 (0.0)	2 (1.2)	1 (0.6)	9 (5.6)**	**0.0 to 1.9**
# 7th cervical centrum: unossified.	2 (9.1)	2 (9.1)	4 (18.2)	7 (30.4)	**0.0 to 41.7**	6 (3.8)	7 (4.2)	4 (2.5)	11 (6.8)	**0.0 to 12.2**
# 5th sternebra: incomplete ossification.	10 (45.5)	11 (50.0)	7 (31.8)	12 (52.2)	**19.0 to 70.8**	17 (10.8)	21 (12.7)	8 (5.0)*	24 (14.8)	**3.1 to 19.9**
13th costal cartilage (uni): short.	0 (0.0)	0 (0.0)	0 (0)	2 (8.7)	**0.0 to 0.0**	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.2)	**0.0 to 0.0**

* Denotes statistically significantly different from control at the *$p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from page 32 of the study report.
# denotes that statistical analysis was conducted on the observations.

III. CONCLUSION

Under the conditions of this study, there were no test substance-related effects on maternal and fetal survival, reproductive outcomes, or gross necropsy findings.

The study investigator considered the NOEL for maternal toxicity to be 15 mg/kg bw/day (a NOAEL was not established by the study investigator). While a decreased body weight gain was noted in 50 mg/kg bw/day maternal animals during the GD 6–8 period, the similarity in corrected body weight gain and overall body weight gain with controls and the lack of any other adverse clinical signs of toxicity suggest that the decreased body weight gain observed is also unlikely to be toxicologically adverse.

Therefore, the primary evaluator considers the maternal toxicity NOAEL to be 50 mg/kg bw/day, based on effects at 150 mg/kg bw/day comprising a salivation incidence of 20/23 animals during GD 11–21 versus an absence of this finding in controls, a lower gestational bodyweight gain in females resulting in a 16% lower corrected bodyweight gain, and statistically significantly decreased food consumption over GD 6 to 12.

The study investigator considered the NOEL for developmental toxicity to be 50 mg/kg bw/day (a NOAEL was not established by the study investigator). The primary evaluator considers that the developmental toxicity NOAEL to be 50 mg/kg bw/day, and the LOAEL is 150 mg/kg bw/day based on increased incidence of incomplete parietal and hyoid centrum skull bone ossification . While a very slight decrease in fetal body weights (-2–3% vs controls) was noted at 150 mg/kg bw/day, the evaluator did not consider this decrease an adverse effect in this case, noting the lack of statistical significance for the effect.

This study is fully reliable (acceptable/guideline) and satisfies the requirements for a developmental toxicity study in rats (OPPTS 870.3700; OECD 414; EC Directive 2004/73 Method B.31; MAFF 12 Nousan 8147).

Appendix A

SA 08347

MEAN MATERNAL BODY WEIGHTS (G) OF ALL PREGNANT FEMALES

	GROUP 1	GROUP 2	GROUP 3	GROUP 4
Maternal Body Weights (gd 0)	255.9 +/- 14.45 N=22	257.4 +/- 15.64 N=22	256.6 +/- 12.16 N=22	257.7 +/- 13.76 N=23
Maternal Body Weights (gd 6)	285.2 +/- 19.10 N=22	282.7 +/- 19.38 N=22	284.2 +/- 14.38 N=22	286.3 +/- 14.89 N=23
Maternal Body Weights (gd 8)	291.1 +/- 19.51 N=22	288.6 +/- 19.47 N=22	287.2 +/- 13.71 N=22	280.6 +/- 15.83 N=23
Maternal Body Weights (gd 10)	300.2 +/- 20.11 N=22	296.0 +/- 20.20 N=22	295.8 +/- 14.13 N=22	287.5 +/- 14.52 N=23
Maternal Body Weights (gd 12)	308.1 +/- 19.95 N=22	304.8 +/- 20.87 N=22	304.0 +/- 14.39 N=22	297.2 +/- 15.70 N=23
Maternal Body Weights (gd 14)	317.0 +/- 19.66 N=22	313.1 +/- 20.97 N=22	311.9 +/- 15.23 N=22	307.0 +/- 16.46 N=23
Maternal Body Weights (gd 16)	331.0 +/- 19.29 N=22	327.3 +/- 20.78 N=22	328.9 +/- 16.52 N=22	322.0 +/- 16.29 N=23
Maternal Body Weights (gd 18)	358.0 +/- 19.02 N=22	356.9 +/- 23.49 N=22	356.8 +/- 18.71 N=22	348.7 +/- 19.93 N=23
Maternal Body Weights (gd 21)	401.6 +/- 23.51 N=22	401.8 +/- 30.85 N=22	404.3 +/- 23.98 N=22	394.4 +/- 25.43 N=23

Data excerpted from the study report, pages 47-48.

Primary review: Australia
Secondary review: US EPA & PMRA

Report: Kennel P (2012) BYI02960: Developmental toxicity study in the rabbit by gavage. Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France. Report No. SA 10314. 26 January 2012. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N°TXRVP090. MRID 48844117. PMRA 2239577. Unpublished.

Kennel P (2012) BYI02960: Range –finding study for developmental toxicity study in the rabbit by gavage. Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France. Report No. SA 10220. 16 September 2013. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: TXRVP023. MRID 49113601. Unpublished.

Guidelines: OPPTS 870.3700 (1998)
OECD 414 (2001)
EC Directive 2004/73 Method B.31 (2004)
MAFF 12 Nousan 8147 (2000)

Deviations: To maintain consistency with conditions of the breeding facility from which they were obtained, animals were kept under a 16 hour light/8 hour darkness light cycle instead of a 12 hour cycle.
Due to experimental error, test substance was suspended in vehicle at w/w percentages instead of w/v. Deviations not expected to compromise study validity.

GLP: Yes Signed statements of GLP, Quality Assurance, and Data Confidentiality were presented in the report.

Executive summary:

In a developmental toxicity study (MRID 48844117) by oral gavage dosing, time-mated female NZW (Crl: KBL) rabbits in groups of 23/dose received BYI02960 (96.2% purity) in 0.5% aqueous methylcellulose 400 vehicle at doses of 0, 7.5, 15, or 40 mg/kg bw/d in dosing volumes of 4 mL/kg bw/d between gestation days (GD) 6–28. For the dams, the following parameters were evaluated: bodyweight, bodyweight gain, food consumption, survival, clinical signs, reproductive outcomes, liver weight and gross pathology. Foetal parameters evaluated were the following: foetal bodyweight, incidences of dead foetuses, and external, visceral, head, and skeletal malformations and variations.

In the main study, there were no test substance-related effects on maternal clinical signs, reproductive parameters litter data endpoints, or gross necropsy findings. However, during GD 6–8, decreased corrected bodyweight gain and decreased food consumption were observed in 40 mg/kg bw/d maternal animals. However, the absolute body weights of the treated and the controls groups were comparable. The decreases in body weight gain and in the food consumption were marginal; these observations were considered not to be toxicologically adverse in this case. **Therefore, the maternal NOAEL was established at 40 mg/kg bw/d, based on no adverse effects were seen at the highest dose tested. When the data of this main study were considered in combination with those of the range finding study (MRID 49113601) (Attachment: Abbreviated Review of the Range Finding study), a maternal LOAEL was established at 80 mg/kg bw/d based on the markedly decrease in food consumption starting GD 6-10 and persisted at lower magnitude to the end of the study and clinical signs (no feces or few feces over several days).**

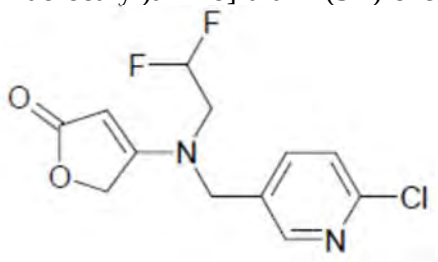
As no evidence of developmental toxicity was observed, and no evidence of an increased frequency of treatment-related, toxicologically adverse external, visceral or skeletal malformations and variations were found; **the developmental toxicity, NOAEL wasis 40 mg/kg bw/d, the highest dose tested in this study**. However, at 80 mg/kg bw/d in the range finding study, there was an increase in the number of dead fetuses (7 fetuses in 4 litters) relative to the controls (2 fetuses in 1 litter). This finding demonstrated an increase in the percent of dead fetuses per litter (80 mg/kg/d: 7.8% vs Control: 1.9%). This increase was reported to be outside of the historical control range of the performing laboratory (0.74 – 6.58%). **Therefore the LOAEL for developmental toxicity could be established as 80 mg/kg bw/d.**

This study is fully reliable (acceptable/guideline) and satisfies the requirements for a developmental toxicity study in rabbits **when considered with the results from the range-finding study (**OPPTS 870.3700; OECD 414; Directive 87/302/EEC B.31; MAFF 12 Nousan 8147).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI02960
   4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one



Batch #:	2009-000239
Purity:	96.2% (method No. 2960C01; Jan 16 2009. Retest Jan 14 2011)
Description:	Beige powder
CAS #:	951659-40-8
Stability of test compound:	Stored in an air-tight, light-resistant container at room temperature. Stated to be stable for two years at 25±5ºC. Batches of the test substance in dosing vehicle were prepared six times during the study, and stored at 5±3ºC when not in use. The study authors cited Report No. SA 07025 (Amir Tahmasseb, 2007) where the test substance was stated to be stable in aqueous methylcellulose at 1 and 250 g/L concentrations over 28 days at room temperature. In the current study, test compound was confirmed to have been mixed homogeneously in vehicle for the F1 preparation, and was present at 90 to 103% of nominal concentrations across the six preparations (F1–F6).

2. Vehicle : 0.5% aqueous methylcellulose 400
3. Test animals

Species:	Rabbit (female, time-mated)
Strain:	NZW (Crl: KBL)
Age at dosing:	Approximately 18±1 weeks old
Weight at dosing:	Approximately 3.04 to 3.86 kg on GD 3 (females)
Source:	Charles River Laboratories, Chatillon-surChalaronne, France, on GD 1 or GD 2
Acclimation period:	4 or 5 days prior to dosing (GD 6)
Diet:	110 C-10 pelleted animal diet from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) *ad libitum*.
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Individually in stainless steel, wire-mesh cages.

4. Environmental conditions

Temperature:	17–21°C
Humidity:	40–70%

Air changes:	10 to 15 per hour
Photoperiod:	16 hour light/8 hour dark cycle (see 'deviations' for explanation)

B. STUDY DESIGN

1. Experimental start/completion

   17 December 2010 to 7 November 2011

2. Animal assignment and treatment

   A total of 160 time-mated female rabbits were received from the supplier on GD 1 or GD 2, with the day of presumed mating recorded as GD 0. On each day of mating, animals were randomised to dosing groups using XMS Path/Tox Version 4.2.2 software program such that differences in group mean bodyweights were minimised. Where possible, dams mated with the same male were not randomised to the same group.

   The dosing regimen was based on the results of a preliminary dose finding study (Kennel 2010; SA 10220) in rabbits where dosing at 80 mg/kg bw/d from GD 6 to 28 induced clinical signs of toxicity in dams, decreased food consumption, bodyweight loss, increased foetal mortality, and decreased foetal bodyweight.(Attachment) The next lowest dose tested of 40 mg/kg bw/d induced only slight bodyweight loss between GD 6 to 8, and the lowest dose tested of 15 mg/kg bw/d produced no evidence of toxicity.

   Based on the results from the preliminary study, doses of 0, 7.5, 15, or 40 mg/kg bw/d BYI02960 in 0.5% aqueous methylcellulose 400 vehicle (4 mL/kg bw dose volume) were selected for the main study (Table IKKA 5.6.11/01-1). BYI02960 was administered to dams on GD 6 to 28. Dosing volumes were based on the most recent body weight. Negative controls received 0.5% aqueous methylcellulose only. On GD 29, all surviving dams were sacrificed by *i.v.* injection of sodium pentobarbital.

Table KIIA 5.6.11/01-1 Study design

Group no. [N=23/group]	Dosing duration (GD)	Dose (mg/kg bw/d)[a]	Test formulation concentration (g/L)[b]	Animal ID No.'s
1	GD 6 to 28	0	0	UR1F1032 to 1054
2		7.5	1.875	UR2F1055 to 1077
3		15	3.75	UR3F1078 to 1100
4		40	10	UR4F1101 to 1123

Final dosing volumes were 4 mL/kg bw for all groups. All surviving dams were sacrificed on GD 29 by i.v. injection of sodium pentobarbital. The day of presumed mating was denoted GD 0. Data excerpted from page 16 of the study report.

3. Dosing suspensions, preparation, and analysis

   Suspensions of test substance (w/w) in 0.5% aqueous methylcellulose 400 were prepared six times for each dosing level during the study, with each batch (F1–F6) of 1.8 L volume. The test substance concentrations in the formulation were 1.875, 3.75, and 10 g/L for the 7.5, 15, and 40 mg/kg bw/d nominal dosing concentrations, respectively. When not in use, the suspensions were stored at 5°C±3°C, and prior to use, suspensions were mixed continuously before and during dosing with an electromagnetic stirrer.

Homogeneity analysis using HPLC was undertaken on the top, middle, and bottom layer of the first batch of the low and high dosing formulations (Table KIIA 5.6.11/01-2). Additionally, concentration analysis using HPLC was undertaken on surface layer samples of the 3.75 g/L F1 formulation, and in all dosing concentrations of the F2–F6 batches. The achieved mean concentrations in the dosing suspension were found to be between 90% (F3 and F4 batches at 10 g/L) to 103% (1.875 g/L F3 batch) of nominal concentrations, and therefore met the laboratory's in-house criteria of 90 to 110% of nominal concentrations.

Stability analysis was not undertaken, with the study author's instead citing Report No. SA 07025 (Kennel, 2007) where the test substance was stated to be stable in aqueous methylcellulose at 1 and 250 g/L concentrations over 28 days at room temperature.

Table KIIA 5.6.11/01-2: Homogeneity/achieved concentration analysis.

Batch ID	Date of analysis (date of formulation)	Layer obtained from batch (H, M, B)	Achieved concentration (g/L)	% of nominal (and per cent St Dev)
0 g/L				
F1	6 Jan, **2011**	H	<VL	-
F2	6 Jan, **2011**	H	<VL	-
F3	14 Jan 2011	H	<VL	-
F4	14 Jan 2011	H	<VL	-
F5	25 Jan, 2011	H	<VL	-
F6	25 Jan, 2011	H	<VL	-
1.875 g/L				
F1	6 Jan, **2011**	H	1.87	100
		M	1.83	98
		B	1.79	95
		H to B Mean	**1.83±0.04**	**97.7±2.5**
F2	6 Jan, **2011**	H	1.830	**98**
F3	14 Jan 2011	H	1.93	**103**
F4	14 Jan 2011	H	1.90	**101**
F5	25 Jan, 2011	H	1.82	**97**
F6	25 Jan, 2011	H	1.85	**99**
3.75 g/L				
F1	6 Jan, **2011**	H	3.61	**96**
F2	6 Jan, **2011**	H	3.70	**99**
F3	14 Jan 2011	H	3.69	**98**
F4	14 Jan 2011	H	3.52	**94**
F5	25 Jan, 2011	H	3.57	**95**
F6	25 Jan, 2011	H	3.75	**100**
10 g/L				
F1	6 Jan, **2011**	H	9.47	95
		M	9.41	94
		B	9.70	97
		H to B Mean	**9.527±0.153**	**95.3±1.5**
F2	6 Jan, **2011**	H	9.31	**93**
F3	14 Jan 2011	H	8.962	**90**
F4	14 Jan 2011	H	9.032	**90**
F5	25 Jan, 2011	H	9.65	**97**
F6	25 Jan, 2011	H	9.83	**98**

'VL' – denotes verifiable limit, equal to 0.1 g/L. 'H' – denotes surface layer, 'M' denotes middle, and 'B' denotes bottom layer. Data excerpted from pages 234 to 238 of the study report.

4. Statistics

The statistical methods employed are presented in Table KIIA 5.6.11/01-3, and proceeded using the program TERATEST Phase 1, V. 12, or SAS programs (Version 9.2). Statistical analyses were performed separately for all pregnant females and for all pregnant females with live foetuses, and as the results were similar only tables for pregnant animals were provided. Group means were compared at the $p \leq 0.05$ and $p \leq 0.01$ levels. Body weight data measured on different days throughout gestation were not statistically analysed, with only descriptive statistics being presented.

Foetal mortality was analysed both using the foetus as the statistical unit and using the litter as the statistical unit.

Table KIIA 5.6.11/01-3. Statistics used in data analyses

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant (p>0.05)	**If preliminary test is significant (p≤0.05)**
Bodyweight change and corrected for gravid uterus weights. No. of corpora lutea, implantation sites, and	**Bartlett Test**	Analysis of variance followed by 2-sided **Dunnett's test if significant**	**Kruskal-Wallis test** followed by 2-sided **Dunn's test if significant**
Mean food consumption Liver weights Pre- and post-implantation loss	**Bartlett Test**	Analysis of variance followed by 2-sided **Dunnett's test if significant**	**Bartlett Test** on Log transformed[A] data**,** if significant then **Kruskal-Wallis test** followed by 2-sided **Dunn's test if signficant;** Otherwise **ANOVA, followed by Dunnett tests** on transformed data if significant
Foetal bodyweight	**Levene Test**	Analysis of variance followed by 2-sided Dunnett's test if significant	Levene Test on Log transformed data, if significant then Kruskal-Wallis test followed by 2-sided Dunn's test if significant; Otherwise ANOVA, followed by Dunnett tests on log transformed data if significant
Sex ratio	**Chi-square test**		
Foetal mortality	**2-sided Fisher Exact test**		
Foetal Skeletal observations	**Fisher Exact test (one-sided)**		

[A] – except for implantation loss, which was transformed using arcsine root transformation.

C. METHOD

1. Observations

   From GD 2 to GD 29, dams were observed daily for clinical signs, and twice daily for mortality and morbidity (once daily on weekends/public holidays). Any abnormal findings were recorded.

2. Body weights

   All dams were weighed on GD 0, 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29.

3. Food consumption

   Food consumption for dams were measured by comparing full and empty feeder weights at GD 3 (full feeder), 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 (full and empty feeder) and 29 (empty feeder only). Instances of food spillage were also recorded.

4. Sacrifice and pathology

At termination (GD 29), surviving dams were sacrificed by *i.v.* injection of sodium pentobarbital, and subjected to gross necropsy including examination of visceral organs and recording of gravid uterus weights. The liver of each pregnant dam was weighed, and a portion retained and fixed in 10% neutral buffered formalin for potential histopathological examination (the liver was not examined). The reproductive tract of each pregnant rat was removed, weighed, and dissected to permit examination of the contents. Uterine horn(s) without visible implantations were immersed in a 20% solution of ammonium sulfide to assist in identification of implantation sites.

5. Reproductive outcomes

The following reproductive parameters were evaluated: Pregnancy rate, premature delivery rate, total resorption rate, mean number of implantations, live foetuses, dead foetuses, corpora lutea counts, and pup sex ratio. Intra-uterine mortality was classified as either an early or late resorption based on the criteria of Gleich & Frohberg (1977). Specifically, early resorption occurred when macroscopic discrimination between fetal residues and placental material was not possible, and late resorption occurred when distinct macroscopic discrimination between foetal and placental tissue was possible. Foetal death was classified when dead conceptuses had distinct digits on fore-paws and hind-paws.

6. Evaluation of fetuses

Parameters evaluated in foetuses were mean live and dead bodyweight, and incidences of external, visceral, head, and skeletal malformations and variations. All live foetuses were sacrificed by subcutaneous injection of sodium pentobarbital, and were counted, weighed, and externally examined. The heads of foetuses from approximately half of each litter were immersed in Bouin's fluid and the internal structures examined after fixation. The bodies of all foetuses were dissected for examination of soft tissue anomalies and sex determination, and they were then eviscerated, skinned, fixed in absolute ethanol and stained using a modification of the Staples and Schnell technique, and examined for skeletal abnormalities.

II. RESULTS AND DISCUSSION

A. MATERNAL EFFECTS

1. Clinical signs of toxicity

There were no test substance related clinical signs at any dose. One control group female (Animal No. UR1F1048) aborted on GD 25, with necropsy findings identifying red foci on lungs and prominent liver lobulation.

2. Mortality

Asides from the control group female that aborted, there was no unscheduled mortality.

3. Body weight, body weight gain, and food consumption

At 7.5 or 15 mg/kg bw/d, there were no effects on bodyweight gain or bodyweights (Table KIIA 5.6.11/01-4).

At 40 mg/kg bw/d, a 12% lower bodyweight gain occurred over GD 6–29 when excluding gravid uterus weight (Table KIIA 5.6.11/01-4). This finding was largely due to a 67% lower ($p \leq 0.05$) bodyweight gain over GD 6–10 relative to controls. The absolute body weights of the compound treated test animals showed no effect (Table KIIA 5.6.11/01-5), and the changes in the body weight gain was within the historical control at practically all the measuring intervals. Therefore, the effects seen at 40 mg/kg bw/day were not adverse.

Table KIIA 5.6.11/01-4 Maternal bodyweight gain (g)

	Dose (mg/kg bw/d)				HC
	0	7.5	15	40	
No. pregnant dams	**23**	**22**	**23**	**22**	
GD 3 to 6 (predose)	0.03±0.076	0.03±0.073 (0%)	0.01±0.075 (-67%)	0.02±0.070 (-33%)	-0.067 to 0.046
GD 6 to 8:	0.02±0.042	0.02±0.035 (0%)	0.00±0.031 (-100%)	-0.01±0.060 (-150%)	-0.026 to 0.025
GD 8 to 10:	0.04±0.046	0.04±0.033 (0%)	0.03±0.025 (-25%)	0.03±0.039 (-25%)	-0.003 to 0.048
GD 10 to 14:	0.03±0.081	0.09±0.056** (+200%)	0.07±0.049* (+133%)	0.06±0.054 (+100%)	0.023 to 0.088
GD 14 to 18:	0.03±0.075	0.05±0.054 (+67%)	0.07±0.045 (+133%)	0.04±0.049 (+33%)	0.046 to 0.101
GD 18 to 22:	0.04±0.046	0.05±0.046 (+25%)	0.03±0.042 (-25%)	0.06±0.061 (+50%)	0.031 to 0.083
GD 22 to 26	0.06±0.080	0.04±0.067 (-33%)	0.04±0.050 (-33%)	0.07±0.058 (+17%)	0.00 to 0.087
GD 26 to 29	0.06±0.069	0.04±0.059 (-33%)	0.03±0.049 (-50%)	0.04±0.076 (-33%)	-0.002 to 0.065
GD 6 to 29 ***% Δ control***	0.28±0.228	0.31±0.111 (+11%)	0.28±0.160 (0%)	0.28±0.116 (0%)	0.221 to 0.439
Corrected BW gain	-0.25±0.177	-0.24±0.110 (+4%)	-0.25±0.157 (0%)	-0.28±0.126 (-12%)	-0.25 to -0.08

* Denotes statistically significantly different from control at the *$p \leq 0.05$ or ** $p \leq 0.01$ level. 'Corrected BW'– denotes bodyweight minus gravid uterus weight. 'HC' – denotes historical control data. Data excerpted from page 28 of the study report, with the exception of per cent change from control which was calculated by the evaluator using Microsoft Excel.

Decreased food consumption of 20% over GD 6 to 8 ($p \leq 0.01$) and 11% over GD 8 to 10 (not statistically significant) was also observed at 40 mg/kg bw/d (Table KIIA 5.6.11/01-5), and was likely linked with the decreased body weight gain observed. The statistically significant increased food consumption in 7.5 and 15 mg/kg bw/d animals between GD 14–18 was considered incidental to treatment.

Table KIIA 5.6.11/01-5 Mean Maternal body weights (kg)

	GROUP 1	GROUP 2	GROUP 3	GROUP 4
Maternal Body Weights (gd 3)	3.52 +/- 0.172 N=23	3.53 +/- 0.224 N=22	3.53 +/- 0.144 N=23	3.52 +/- 0.180 N=22
Maternal Body Weights (gd 6)	3.55 +/- 0.150 N=23	3.56 +/- 0.213 N=22	3.54 +/- 0.144 N=23	3.53 +/- 0.181 N=22
Maternal Body Weights (gd 8)	3.56 +/- 0.142 N=23	3.57 +/- 0.216 N=22	3.55 +/- 0.147 N=23	3.52 +/- 0.191 N=22
Maternal Body Weights (gd 10)	3.61 +/- 0.156 N=23	3.61 +/- 0.227 N=22	3.57 +/- 0.158 N=23	3.55 +/- 0.210 N=22
Maternal Body Weights (gd 12)	3.62 +/- 0.159 N=23	3.65 +/- 0.251 N=22	3.60 +/- 0.160 N=23	3.57 +/- 0.206 N=22
Maternal Body Weights (gd 14)	3.64 +/- 0.173 N=23	3.70 +/- 0.250 N=22	3.65 +/- 0.167 N=23	3.61 +/- 0.206 N=22
Maternal Body Weights (gd 16)	3.65 +/- 0.181 N=23	3.74 +/- 0.266 N=22	3.69 +/- 0.178 N=23	3.63 +/- 0.201 N=22
Maternal Body Weights (gd 18)	3.67 +/- 0.187 N=23	3.75 +/- 0.272 N=22	3.71 +/- 0.189 N=23	3.64 +/- 0.209 N=22
Maternal Body Weights (gd 20)	3.68 +/- 0.194 N=22	3.77 +/- 0.262 N=22	3.73 +/- 0.193 N=23	3.67 +/- 0.195 N=22
Maternal Body Weights (gd 22)	3.71 +/- 0.198 N=23	3.80 +/- 0.262 N=22	3.75 +/- 0.202 N=23	3.71 +/- 0.193 N=22
Maternal Body Weights (gd 24)	3.74 +/- 0.231 N=23	3.82 +/- 0.257 N=22	3.78 +/- 0.218 N=23	3.74 +/- 0.191 N=22
Maternal Body Weights (gd 26)	3.77 +/- 0.238 N=22	3.83 +/- 0.254 N=22	3.79 +/- 0.224 N=23	3.77 +/- 0.180 N=22
Maternal Body Weights (gd 29)	3.83 +/- 0.269 N=22	3.87 +/- 0.254 N=22	3.83 +/- 0.240 N=23	3.81 +/- 0.209 N=22

Group 1= 0 ppm; Group 2= 7.5 mg/kg/day; Group 3= 15 mg/kg/day; Group 4= 40 mg/kg/day
Table excerpted from pages 46-48 of the study report.

Table KIIA 5.6.11/01-6: Mean food consumption (g/d) in dams (pregnant only)

	Dose (mg/kg bw/d)			
	0	**7.5**	**15**	**40**
No. of pregnant dams	**22**	**22**	**23**	**22**
GD 3 to 6:	165.3±24.04	171.6±28.85 (+4%)	161.7±32.45 (-2%)	153.6±43.55 (-7%)
GD 6 to 8:	173.9±24.13	169.6±20.60 (-2%)	162.3±28.15 (-7%)	139.0±54.01** (-20%)
GD 8 to 10:	173.3±18.07	177.8±30.24 (+3%)	171.6±23.09 (-1%)	153.6±48.73 (-11%)
GD 10 to 14:	148.6±31.20	163.9±32.94 (+10%)	153.4±35.36 (+3%)	136.9±42.11 (-8%)
GD 14 to 18:	122.6±41.92	155.8±29.56* (+27%)	149.6±36.65* (+22%)	121.8±39.3 (-1%)
GD 18 to 22:	132.2±40.91	152.1±28.34 (+15%)	146.9±34.94 (+11%)	137.3±28.34 (+4%)
GD 22 to 26:	100.5±35.52	107.8±35.28 (+7%)	101.5±34.91 (+1%)	110.9±21.60 (+10%)
GD 26 to 29:	99.2±36.87	99.1±30.02 (0%)	90.2±35.58 (-9%)	94.3±28.55 (-5%)

* Denotes statistically significantly different from control at the *$p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from pages 54 to 55 of the study report, with the exception of per cent change from control which was calculated by the evaluator using Microsoft Excel.

4. Reproductive outcomes

There were no test substance-related effects on reproductive parameters or litter data endpoints, including number of live foetuses per litter, number of implant sites per dam, percentages of pre- and post-implantation losses, early or late resorptions, foetal death status, or sex ratio (Table KIIA 5.6.11/01-6). Overall pregnancy rates ranged from 96% at 7.5 and 40 mg/kg bw/d, and 100% in control and 15 mg/kg bw/d groups. The increase in implantation sites in 40 mg/kg bw/d animals, though outside historical controls, was accompanied by a proportional increase in live foetuses, and not considered a toxicologically adverse effect.

5. Gross pathology

Necropsy findings in dams were unremarkable.

Table KIIA 5.6.11/01-7: Reproductive parameters

	Dose (mg/kg bw/d)				HC
	0	**7.5**	**15**	**40**	
Maternal data					
No. Animals assigned	23	23	23	23	NA
No. Animals pregnant	23	22	23	22	NA
Pregnancy rate (%)	100	96	100	96	NA
No. Animals non-pregnant	0	1	0	1	NA
Maternal wastage					
No. died (total)	0	0	0	0	NA
No. died pregnant	0	0	0	0	NA
No. died non-pregnant	0	0	0	0	NA
No. premature delivery	0	0	0	0	NA
No. abortions	1	0	0	0	NA
Uterine data					
Total No. corpora lutea[c]	253	280	265	283	NA
Corpora lutea / dam	11.5±1.8	12.7±1.9	11.5±1.6	12.9±2.6	10.79 to 13.21
Total No. implantations[c]	231	239	235	254	NA
Implantations / dam	10.5±2.1	10.9±2.3	10.2±2.0	11.5±2.9	9.10 to 10.95
Total No. litters[c]	22	22	23	22	NA
Total No. live fetuses[c]	213	223	216	228	NA
Live foetuses / dam[c]	9.7±2.5	10.1±2.1	9.4±1.8	10.4±2.4	8.23 to 9.90
Total No. dead fetuses	8	5	7	10	NA
Dead foetuses / dam[c] (%)	3.2±6.8	2.0±4.3	2.9±5.4	3.8±6.0	0.74 to 6.58
Early resorptions[c]	7	9	9	13	NA
Late resorptions[c]	3	2	3	3	NA
Early resorptions / dam	0.3±0.8	0.4±0.7	0.4±0.5	0.6±0.6	0.167 to 1.000
Late resorptions / dam	0.1±0.5	0.1±0.3	0.1±0.5	0.1±0.5	0.000 to 0.333
Litters with total resorpt.[c]	0	0	0	0	NA
Preimplantation loss per litter (%)	9.2±8.5	14.5±13.6	11.0±14.1	10.9±11.0	9.08 to 26.73
Postimplantation loss per litter (%)	8.7±13.0	6.1±9.2	7.4±9.2	9.7±9.5	4.07 to 19.25
Mean foetal weight, M &F (g)	37.23±6.20	38.07±3.44	39.14±4.26	37.89±4.69	34.96 to 41.37
Mean foetal weight, M (g)	37.31±6.49	38.66±4.05	39.48±4.50	38.04±5.77	34.92 to 42.33
Mean foetal weight, F (g)	36.52±6.86	37.60±3.54	38.81±4.41	37.75±4.57	34.57 to 40.85
Sex ratio (% male)	48.4±18.6	52.4±15.2	50.0±16.4	47.4±20.7	44.0 to 53.8

'c' – denotes that statistical analysis was not conducted for that parameter. No findings were statistically significant ($p \leq 0.05$). 'HC' – denotes historical control. 'NA' denotes not applicable. Data excerpted from page 30 of the study report.

B. FETAL EFFECTS

Foetal bodyweights were unaffected by treatment. There were no test substance-related effects on the incidence of external malformations or variations..

Five foetuses in five different litters were observed with external malformations: three in the low dose group and two in the control group. As they were observed with no dose-relationship, the study author regarded these malformations to be unrelated to the test substance.

At 40 mg/kg bw/d, the incidence of the external variation "eye bulge (unilateral/bilateral): protruding" was higher than in the control group and was outside the range of in-house historical controls in terms of foetal incidence. However, noting the observation was recorded in only one litter and not corroborated with the internal and skeletal observations, it is likely the effect is unrelated to the test substance.

No treatment-related changes in the frequency of visceral and skeletal malformations and variations were observed. Three types of delayed/incomplete ossification variations in hyoid centrum, 5th/6th sternebrae and pubis (unilateral/bilateral) were noted by the study author to be above control incidences, but based on their presence within historical control ranges and lack of statistical significance, were regarded to be unrelated to the test substance (Table KIIA 5.6.11/01-8). Additionally, a slight variation in the frequency of the insertion point (unilateral/bilateral) of the pelvic girdle on the 2nd sacral vertebra was noted, though reported values did not show a dose relationship and were considered comparable to historical controls by both litter and foetal values. The study evaluator agreed that the reported skeletal variations were not treatment-related or toxicologically adverse in this case.

Table KIIA 5.6.11/01-8: Selected findings from skeletal examination

Observation	Dose (mg/kg bw/d)				HC range (%)	Dose (mg/kg bw/d)				HC range (%)
	0	7.5	15	40		0	7.5	15	40	
	No. litters examined					No. foetuses/heads examined				
	22	22	23	22		213/112	223/117	216/115	228/119	
	No. of litters affected (% affected)					No. of foetuses affected (% affected)				
Hyoid centrum: incomplete ossification or unossified	9 (40.9)	10 (45.5)	11 (47.8)	12 (54.5)	**4.8 to 63.6**	20 (17.9)	13 (11.1)	16 (13.9)	23 (19.3)	**1.1 to 28.6**
# 5th and 6th sternebra: incomplete ossification.	8 (36.4)	8 (36.4)	7 (30.4)	10 (45.5)	**14.3 to 45.8**	18 (8.5)	11 (4.9)	10 (4.6)	19 (8.3)	**2.1 to 12.6**
Pubis (uni/bi): incomplete ossification	1 (4.5)	2 (9.1)	0 (0.0)	3 (13.6)	**0.0 to 27.3**	2 (0.9)	2 (0.9)	0 (0.0)	5 (2.2)	**0.0 to 4.3**
Insertion point (uni/bi) of pelvic girdle on 2nd sacral vertebra	21 (95.5)	22 (100)	19 (82.6)	17 (77.3)	**66.7 to 95.8**	85 (39.9)	87 (39.0)	85 (39.4)	95 (41.7)	**28.2 to 56.7**

No observations were statistically significantly different from control at the $p \leq 0.05$ or $p \leq 0.01$ level. Data excerpted from page 32 of the study report

III. CONCLUSION

Under the conditions of this study, there were no test substance-related effects on maternal clinical signs, reproductive parameters or litter data endpoints, gross necropsy findings. For fetuses, there were no test substance-related effects on bodyweights, sex ratio, or the incidence of malformations or external variations.

During GD 6–8, decreased corrected bodyweight gain and decreased food consumption were observed in 40 mg/kg bw/day maternal animals. However, the absolute body weights of the treated and the controls groups were comparable. The decreases in body weight gain and in the food consumption were marginal; these observations were considered not to be toxicologically adverse in this case. Therefore, **the maternal NOAEL was established at 40 mg/kg bw/dy, based on no adverse effects were seen at the highest dose tested**.**When the data of this main study were considered in combination with those of the range finding study (MRID 49113601) (Attachment: Abbreviated Review of the Range Finding study), a maternal LOAEL could be established at 80 mg/kg bw/d based on the markedly decrease in food consumption starting GD 6-10 and persisted at lower magnitude to the end of the study and clinical signs (no feces or few feces over several days).**

As no evidence of developmental toxicity was observed, and no evidence of an increased frequency of treatment-related, toxicologically adverse external, visceral or skeletal malformations and variations were found; the developmental toxicity**, NOAEL was 40 mg/kg bw/d, the highest dose tested in this study**. However, at 80 mg/kg bw/d in the range finding study, there was an increase in the number of dead fetuses (7 fetuses in 4 litters) relative to the controls (2 fetuses in 1 litter). This finding demonstrated an increase in the percent of dead fetuses per litter (80 mg/kg/d: 7.8% vs Control: 1.9%). This increase was reported to be outside of the historical control range of the performing laboratory (0.74 – 6.58%). Therefore the **LOAEL for developmental toxicity was established as 80 mg/kg bw/d.**

This study is fully reliable (acceptable/guideline) and satisfies the requirements for a developmental toxicity study in rabbits **when considered with the results from the range-finding study (**OPPTS 870.3700; OECD 414; Directive 87/302/EEC B.31; MAFF 12 Nousan 8147).

Attachment
Abbreviated Review of the Range-Finding study

Reference: Kennel P (2012) BYI02960: Range –finding study for developmental toxicity study in the rabbit by gavage. Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France. Report No. SA 10220. 16 September 2013. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: TXRVP023. MRID 49113601. Unpublished.

Experimental design and procedures

Four groups of 8 time-mated female NZ whilte rabbits (Charles River Laboratories, France) were adminitered BYI 02960 (beige powder, 96.2% w/w purity) by gavage at dose levels of 0, 15, 40, or 80 mg/kg bw/day. Doses were administered daily at a volume of 4 ml/kg bw to each female from GD 6 to 28 based on the most recent recorded animal body weight. The control animals received an equivalent volume of the vwhicle alone (0.5% methylcellulose). The dosages used in this range-finding study were based on results from a previous study where 3 non-pregnant female rabbits were dosed at 50 or 200 mg/kg bw/day for 10 consecutive days. At 200 mg/kg, 2/3 females died on study day 2 and 3. At 50 mg/kg, no mortality was found; no body weight changes was seen in 2/3 females. One 50 mg/kg female had marginally reduced body weight, while food consumption was reducted on a few occations for the 3 animals.

Clinical observations were recorded daily. Maternal body weights were recorded on GD 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Food consumption was measured during the intervals GD 3-4, 4-5, 5-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, 18-20, 20-22, 22-24, 24-26, 26-28 and 28-29. Selected serum chemistry parameters were determined at the end of the study. At scheduled sacrifice, on GD 29, a macroscopic examination of visceral organs was performed, the gravid uterine weight was recorded and the dams were evaluated for number of corpora lutea, number and status of implantations (resorptions, dead and live fetuses). The liver weight was recorded for all pregnant females. A portion of liver was retained in 10% neutral buffered formalin from all females on study for possible histological examination. Liver specimens were not examined and were discarded before completion of the study. Live fetuses were removed from the uterus, counted, weighed, killed and examined externally.

Results

Maternal Findings

Mortality, abortions and clinical signs: There were no, deaths or abortions at any dose level. At 80 mg/kg bw/day, 5/8 dams showed no or few feces over several days while 2/8 controls showed similar signs. No clinical sign was seen in 40 and 15 mg/kg/day females.

Pregnancy Rates: No effect on the pregnancy rate at any dose levels.

Body Weights: There was a slight decrease in mean maternal body weights at 80 mg/kg/day group starting from GD 8 to the end of the study (≈5%) relative to the controls, but the decrease did not show a statistically significance (Table 1). The body weight changes also significantly decreased in GD 6-10 at 80 mg/kg/day (Table 2). At 40 mg/kg/day, there was a decrease in mean body weight changes, but the decrease was not statistically significant.

Table 1. Mean Maternal body weights (kg) of pregnant female rabbits (N=8)				
Gestation day	**Dose (mg/kg bw/day)**			
	0	**15**	**40**	**80**
3	3.48 ± 0.16	3.44 ± 0.14	3.41 ± 0.14	3.44 ± 0.17
6	3.58 ± 0.17	3.47 ± 0.13	3.47 ± 0.12	3.46 ± 0.16
8	3.54 ± 0.18	3.48 ± 0.16	3.43 ± 0.09	3.37 ± 0.17 (↓5%)
10	3.57 ± 0.18	3.51 ± 0.18	3.44 ± 0.09	3.39 ± 0.19 (↓5%)
12	3.60 ± 0.18	3.53 ± 0.14	3.47 ± 0.10	3.42 ± 0.21 (↓5%)
16	3.67 ± 0.16	3.58 ± 0.15	3.52± 0.10	3.45 ± 0.22 (↓6%)
20	3.71 ± 0.21	3.36 ± 0.18	3.58 ± 0.09	3.49 ± 0.27 (↓6%)
24	3.75 ± 0.24	3.67 ± 0.19	3.63 ± 0.09	3.54 ± 0.26 (↓6%)
29	3.81 ± 0.27	3.74 ± 0.22	3.68 ± 0.10	3.64 ± 0.27 (↓5%)

Data excerpted from pages 41-43 of the study report.

Table 2. Mean maternal body weights changes (kg) of pregnant female rabbits (N=8)				
Gestation day	**Dose (mg/kg bw/day)**			
	0	**15**	**40**	**80**
3 – 6	0.06 ± 0.07	0.03 ± 0.03	0.06 ± 0.06	0.02 ± 0.03
6 – 8	0.01 ± 0.03	0.01 ± 0.03	-0.04 ± 0.06	-0.09 ± 0.04**
6 – 10	0.04 ± 0.03	0.04 ± 0.04	-0.03 ± 0.07	-0.07 ± 0.07**
6 – 18	0.17 ± 0.07	0.13± 0.07	0.09 ± 0.10	0.03 ± 0.14
6 – 26	0.22 ± 0.15	0.23 ± 0.13	0.19 ± 0.13	0.11 ± 0.19
6.- 29	0.27 ± 0.17	0.27 ± 0.16	0.21 ± 0.15	0.18 ± 0.21

- *: P≤0.05 **: p≤0.01. Data excerpted from pages 45-47 of the study report.

Table 3. Mean maternal feed consumption (g/day) of pregnant female rabbits (N=8)				
Gestation day	**Dose (mg/kg bw/day)**			
	0	**15**	**40**	**80**
3 – 6	166.8 ± 25.2	152.2 ± 16.5	164.3 ± 22.0	159.0 ± 17.0
6 – 8	156.6 ± 21.7	154.6 ± 16.8	124.1 ± 30.7	53.6 ± 47.1** (↓64%)
8 – 10	172.0 ± 20.9	158.9 ± 15.0	143.9 ± 31.4	99.1 ± 56.4**(↓42%)
10 – 14	149.3 ± 31.8	143.3 ±19.0	135.8 ± 31.9	112.5 ± 41.3(↓25%)
14 – 18	144.9 ± 32.9	144.8 ± 21.4	138.5 ± 25.7	110.8 ± 48.4(↓24%)
18- 22	144.5 ± 39.9	152.8 ± 21.8	140 ± 25.2	114.1 ± 50.9(↓21%)
22 – 26	108.2 ± 41.1	113.4 ± 26.3	107.2 ± 30.7	94.3 ± 20.0(↓13)
26 -29	98.3 ± 24.8	105.2 ± 25.5	94.5 ± 33.2	94.5 ± 25.7(↓4%)

- *: P≤0.05 **: p≤0.01. Data excerpted from pages 49-50 of the study report.

Food consumption: At 80 mg/kg/day, mean maternal food consumption was markedly decreased during the intervals of GD 6-8 and 8-10 (Table 3). The reductions were 64% and 42%, respectively, relative to the controls, and showed statistically significance. The deceases persisted until the end of the study at progressively smaller magnitude. At 40 mg/kg/day, the mean maternal food consumption was reduced also without showing statistical difference relative to the controls. However, despite the reduction in food consumption, the absolute body was only slightly decrease, and the decrease in food consumption might not approach the magnitude of adverse effect.

Developmental Observations: At cesarean section, there was an increase in the number of dead fetuses at 80mg/kg/day compared to the controls (7 fetuses in 4 litters vs 2 fetuses in 1 litter), as also reflected with an increase in the percentage of dead fetuses per litter (7.8 vs 1.9%, outside the range of in-house HCD: 0.74-6.58) (Table 4). Mean fetal body weights were reduced by 8% compared to the controls (34.62 vs 37.57 g, marginally outside the range of in-house HCD: 34.96-41.37), but this decrease was attributable to one litter in presence of marked maternal toxicity (mean of 35.85 g if this animal is excluded). All other litter parameters, including the number of live fetuses per litter, number of implant sites per dam, percentages of pre- and post-implantation losses, early or late resorptions were unaffected by treatment.

At 40 and 15 mg/kg/day, there were no treatment-related findings on developmental parameters.

Table 4. Summary of Developmental Parameters (N=8)

Dose groups (mg/kg/day)	0	15	40	80
No. Corpora Lutea per Dam	11.6 +/- 1.6	11.5 +/- 2.1	11.1 +/- 2.1	11.6 +/- 2.4
No. Implant Sites per Dam	9.4 +/- 2.1	9.5 +/- 1.8	9.1 +/- 2.2	10.3 +/- 2.8
Percent Pre-Implantation Loss per Dam	19.7 +/- 12.2	16.3 +/- 14.3	18.0 +/- 13.8	13.3 +/- 13.1
No. Early Resorption per Litter	0.5 +/- 0.8	0.4 +/- 0.5	0.5 +/- 0.8	0.0 +/- 0.0
No. Late Resorption per Litter	0.0 +/- 0.0	0.3 +/- 0.7	0.0 +/- 0.0	0.0 +/- 0.0
No. Live Fetuses per Litter (a)	8.6 +/- 1.7	8.6 +/- 2.3	8.3 +/- 2.1	9.4 +/- 2.6
No. Live Fetuses (a)	69	69	66	75
No. Litter with Live Fetuses (a)	8	8	8	8
No. Dead Fetuses	2	2	3	7
No. Litter with Dead Fetuses	1	1	3	4
Percent Dead Fetuses per Litter (a)	1.9 +/- 5.4	2.5 +/- 7.1	4.8 +/- 7.4	7.8 +/- 8.9
Percent Post-Implantation Loss per Litter	7.2 +/- 8.2	10.1 +/- 15.0	9.9 +/- 7.6	7.8 +/- 8.9
Fetal Body Weights (g) (a)	37.57 +/- 3.53	39.33 +/- 4.01	38.22 +/- 2.40	34.62 +/- 5.19

(a) : Statistical analysis was not conducted on this endpoint.

Mean value of group was significantly different from control group with Dunnett test of significance * alpha = 0.05 / ** alpha = 0.01

Mean value of group was significantly different from control group with Dunn test of significance + alpha = 0.05 / ++ alpha = 0.01

Data excerpted from pages 59-60 of the study report.

Fetal Observation: No treatment-related external fetal effects were seen at any dose level.

Discussion and Conclusion

In this range-finding developmental toxicity study, time-pregnant rabbits (8/dose group) were administered BYI 02960 by gavage (batch 2009-000239: a beige powder, 96.2% w/w purity) from gestation day (GD) 6 to 28. The doses given were 0, 15, 40 and 80 mg/kg/day in suspension in aqueous solution of 0.5% methylcellulose 400. The volume of administration was 4 ml/kg based on the most recent body weight recorded.

The results showed that at 80 mg/kg/day, BYI 02960 caused slight maternal body weight loss and reduced food consumption, and the effects appeared not to approach the magnitude of adverse effect.

In addition, an increased number and percentage of dead fetuses and a reduction in mean fetal body weight compared to controls were noted at the cesarean section. No treatment-related changes were noted at the external fetal examination.

Therefore, at 80 mg/kg/day, no adverse effects were seen in the dams. No effect on developmental parameters was found at 40 mg/kg/day. At 15 mg/kg/day, there were no treatment-related effects on maternal or developmental parameters.

Primary review: Australia
Secondary review: US EPA & PMRA

Report: IIA 5.6.1/02 Milius AD (2011) Technical Grade BYI 02960: A Two-Generation Reproductive Toxicity Study in the Wistar Rat. Xenometrics, LLC 17745 S. Metcalf Ave. Stilwell, Kansas 66085-9104. Study No 09-R72-SA. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. 17 October 2011. MRID 48844119. PMRA 2239237. Unpublished.

Milius, AD (Technical Grade BYI 02960: A Dose Range-Finding Reproductive Toxicity Study in the Wistar Rat. Xenometrics, LLC, Stilwell, KS. Study Number: 09-P72-RB. November 1, 2010. MRID 48844120. Unpublished.

Guidelines: OECD No. 416 (2001)
OPPTS 870.3800 (1998)
MAFF 12 Nousan 8147 (2000)

Deviations: During lactation day 7 for one lactating P_2 female at 100 ppm, and one to three days of lactaction for between two and ten P_2 females at 100 ppm, dietary concentrations were accidentally not adjusted for lactation period (*i.e.*, double intended dosage). This was unlikely to have affected the reliability of the study results.

GLP: Yes Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a two-generation reproduction study (MRID 48844119), groups of Wistar [(Crl:WI) Han] rats (30/sex/group) received BYI 02960 in the diet (96.2%; BYI 02960-01-03) at concentrations of 0, 100, 500, or 1800 ppm (equivalent to 0, 6.6/7.7, 32.5/38.7, and 117/137 mg/kg bw/day for P_1– M/F premating intakes; and 0/0, 6.4/7.8, 32.0/39.6, and 122.1/143.4 mg/kg bw/day for P_2–M/F premating intakes). The test substance was solvated in acetone prior to incorporation into the diet. Parental animals received the test diet for at least 70 days before the two week mating period. Confirmed pregnant dams then continued to receive admixed diet during gestation (22 days) and lactation to weaning (21 days). During lactation, test substance concentrations were reduced by 50% to account for the corresponding increases in feed intakes of dams during this period. At weaning on LD 21, F_1 pups (30/sex/group) were randomly selected to comprise the P_2 generation and treated in the same manner as their respective P_1 generation sires and dams. Excess F_1 pups were necropsied. P_2 maternal animals were retained until weaning of the subsequent F_2 generation at LD 21.

Under the conditions of the study, BYI 02960 produced no effects on clinical signs, gross necropsy findings, reproductive organs, sperm parameters, or gestation length in both reproductive generations.

In parental observations, clear treatment-related decreases in mean bodyweight (↓10%) with associated reduction in bodyweight gain were observed at 1800 ppm in P_2 females during premating period. Decreased food consumption was noted at 1800 ppm females in both generations during the pre-mating period..

While reproductive parameters were unaffected by treatment in P_1 females, a slight decrease (-17%) in the number of estrus cycles and a slight decrease (-8%) in the total number of implantation sites were noted in 1800 ppm P_2 females, which may be associated with the decreased bodyweights

observed at the same dose. In addition, a slight reduction in epididymal sperm count in P_1 and P_2 males and in testicular sperm count in P_2 males was observed at 1800 ppm.

In terminal observations, treatment-related histopathological changes were noted in the liver (centrilobular hepatocellular hypertrophy) in P_1 males, and accompanied by slight (+9%) increases in the absolute and relative liver weights, though these changes were considered an adaptive effect in the context of this study. While statistically significant increases in absolute and relative liver weights were also noted in other 1800 ppm animals across both generations, no other treatment-related liver histopathological findings were observed. An increase in the relative thyroid weight in both P_1 and P_2 males was not accompanied by gross or histopathological change, and the toxicological significance is uncertain.

Ovarian follicle counts in F_1 females selected as P_2 maternal animals were unaffected by treatment with the test substance.

In fetal observations, general survival parameters were unaffected by treatment in both generations. A slight decrease (-15%) in overall litter size in F_2 pups at 1800 ppm was noted, and when considered along with decreased (-8%) overall implantations in P_2 dams, suggests a sllight effect on reproductive function at 1800 ppm.

Pup body weights were unaffected by treatment at 100 ppm in both generations. Treatment-related and statistically significant decreases in mean bodyweight (-7 to -14%) and bodyweight gain (-8 to -14%) were observed during the weaning period in both generations at 1800 ppm, and in the F_2 generation at 500 ppm. Terminal findings (necropsy, histopathology and organ weight analyses) did not report treatment-related effects in both generations.

Treatment-related changes in sexual maturation were limited to slight delays in vaginal patency and preputial separation at 1800 ppm in F_1 pups; these effects were considered secondary to decreased mean bodyweights in these animals during weaning.

The NOAEL for parental toxicity was 500 ppm (38.7 mg/kg bw/d); the LOAEL was 1800 ppm (137 mg/kg/day) based on decreased bodyweight (↓10%) with associated reduced body weight gains in P_1 females during the pre-mating period.

The **NOAEL for offspring toxicity was 100 ppm (7.7 mg/kg bw/day); LOAEL was 500 ppm (38.7)** based on decreased bodyweight (-7%) in the F_2 male and female pups.

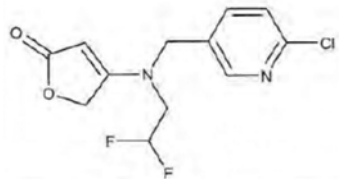
The **NOAEL for reproductive toxicity was 500 ppm (38.7 mg/kg bw/day); LOAEL was 1800 ppm (137 mg/kg/day)** based on a 17% lower ($p \leq 0.05$) number of estrus cycles, In addition, a slight reduction in epididymal sperm count in P_1 and P_2 males and in testicular sperm count in P_2 males was observed.

This study was classified as reliable (**acceptable/guideline**) and satisfied the guideline requirements (OPPTS 870.3800; OECD N0. 416; MAFF 12 Nousan 8147) for a reproduction and fertility study.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
4-[(6-chloropyridin-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one (BYI02960)



Lot/Batch #:	2009-000239
Purity:	96.2% (14 Jan 2011, various methods of analysis)
Description:	Beige powder
CAS #:	Not reported
Stability of test compound:	Stated to be stable for two years if stored at 20±10ºC. The test material was reported to be stable in feed at room temperature over the study period, and homogeneity, achieved concentration, and stability analyses were provided. Batches of admixed diet were prepared weekly for the first three weeks of the study, and monthly thereafter.

2. Vehicle : Acetone vehicle and incorporated into feed
3. Test animals

Species:	Rat
Strain:	Wistar; (Crl:WI) Han
Age at dosing:	P_1 generation approximately 8 to 9 weeks
Weight at dosing:	160–223 g for males; 143–188 g for females
Source:	Charles River Laboratories Inc., Raleigh, NC, USA.
Acclimation period:	Seven days
Diet:	Purina Mills Certified Rodent Diet 5002 in "meal" form, *ad libitum*. During the in-life study phases of premating (10 wks for P_1; 3 to 4 wks for P_2), mating (14 days), gestation (22 days), and lactation to weaning (21 days), test substance was incorporated into the feed of all animals except negative controls.
Water:	Tap water *ad libitum*
Housing:	Individually in suspended stainless steel cages, with deotised cage board in the bedding trays. During gestation and lactation, individual dams (and their litter) were housed in polycarbonate cages with corn-cob bedding. During the mating phase, breeding pairs were housed within a single cage.

4. Environmental conditions

Temperature:	21 to 22.3°C

Humidity:	36–60.4%
Air changes:	Minimum 10 exchanges/hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. In-life initiation/completion

   21 September 2009 to 25 July 2010

2. Animal assignment and treatment

   Animals were assigned to dose groups by computerized, stratified randomisation such that bodyweights were within 20% of the group mean. Once assigned to groups, parental animals were implanted with a chip encoding a unique identifying code, and live born pups were marked with a tattoo.

   Test substance was dissolved in acetone vehicle and incorporated into the feed of all animals except negative controls, which received diets containing the acetone vehicle only.

   Over the pre-mating (10 weeks for both P generations) and mating (14 days) periods, parental animals in groups of 30/sex/dose received diets containing 0, 100, 500, or 1800 ppm BYI02960. After confirmation of successful mating as evidenced by sperm-positive vaginal smear - denoted study day 0 - dams were placed into individual polycarbonate nesting cages. Confirmed pregnant dams then continued to receive admixed diet during gestation (22 days) and lactation to weaning (21 days). During lactation, test substance concentrations were reduced by 50% to account for the corresponding increases in feed intakes of dams during this period. On LD4, each litter with more than eight pups was adjusted by computer randomisation software to yield 4/sex/litter or a close a ratio as possible, with excess pups sacrificed by decapitation and any abnormal carcasses subjected to external examination and necropsy.

   At weaning on LD 21, 30 male and 30 female F_1 rats/group were randomly selected to comprise the P_2 generation and received the same concentration level as their respective P_1 generation sires and dams. Animals selected as P_2 generation parents were maintained for an additional 3–4 weeks after weaning before initiation of the P_2 reproductive generation.

   An additional group of ten males and nine females from the breeder were kept for observation only, and five males and four females were subjected to necropsy at week 4 of the P_1 dosing phase.

   The experimental design is described in Tables KIIA 5.6.1/02-1, KIIA 5.6.1/02-2 and KIIA 5.6.1/02-3. Animal housing and husbandry were carried out in accordance with the provisions in the *Guide to the Care and Use of Laboratory Animals* (1996, National Research Council, Washington, DC). Achieved intakes of the test substance for each phase of the study are shown below. The premating compound intakes for P_1 animals were used for to derive the NOAEL and LOAEL, and they are indicated as underlined and *italicised* font in Table KIIA 5.6.1/02-2 below.

Table KIIA 5.6.1/02-1: Treatment Groups and Dietary Concentrations

P Generation No. per sex	F_1 Generation No. per sex	Dietary concentration (ppm)	
		In life excluding LD	During lactation (females only)
30	30	0	0
30	30	100	50
30	30	500	250
30	30	1800	900

Table KIIA 5.6.1/02-2: Mean test substance intakes (mg/kg bw/d) over study periods

Study phase	Nominal dose (ppm)[A]		
	100	500	1800
Premating (P_1-gen) – **M**	6.6	32.5	117.4
Premating (P_2-gen) – **M**	6.4	32.0	122.1
Premating $\bar{x}$ P_1- and P_2-gen – M	***6.5***	***32.3***	***119.8***
Premating (P_1-gen) – **F**	7.7	38.7	137.0
Premating (P_2-gen) – **F**	7.8	39.6	143.4
Premating $\bar{x}$ P_1- and P_2-gen - F	***7.8***	***39.2***	***140.2***
Gestation (P_1-gen) – **F**	6.9	34.3	134.0
Gestation (P_2-gen) – **F**	7.0	36.6	168.8
Gestation $\bar{x}$ P_1- and P_2	**7.0**	**35.5**	**151.4**
Lactation (P_1-gen) – **F**	7.8	37.4	140.4
Lactation (P_2-gen) – **F**	7.7	42.2	160.3
Lactation $\bar{x}$ P_1- and P_2	**7.8**	**39.8**	**150.4**

[A] – Except during lactation, where nominal dosing concentration were halved to account for increased feed intake from lactating animals. Data excerpted from page 11 of the study report.

Table KIIA 5.6.1/02-3: Sacrifice schedule in two-generation reproduction study

Animals	Generation	Schedule	Final necropsy date
Adult males	P_1, P_2	After siring litters P_1 – study day 106 After siring litters P_2 – test Days 0 to 8 post-mating	22 Feb 2010 (P_1) 7 July 2010 (P_2)
Pregnant females	P_1, P_2	Lactation day 21 – on day of weaning litters	22 Feb 2010 (P_1) 29 Jul 2010 (P_2)
Nonpregnant females	P_1, P_2	24 days after the end of cohabitation	NA
Day 4 culled pups	F_1, F_2	Lactation day 4	NA
Weanlings	F_1, F_2	On day of weaning – postnatal Day 21 (except F_1 rats selected as P_2 parental rats)	22 Feb 2010 (F_1) 7 Apr 2011 (F_2)

'NA' – denotes 'not available'.

3. Dose Selection

The dosing regimen was selected based on a preliminary dose finding dietary study (MRID 48844120), which employed concentrations of 0, 200, 700, and 2000 ppm. At 2000 ppm, marked reduction in bodyweight with associated decreased in body weight gains in parental females, decreased pup weight, and increased liver weights for males were seen. The 700 ppm group showed minor reduction in body weight without statistical significance. No treatment-related effect was observed at 200 ppm.

4. Diet preparation and analysis

Batches of admixed diet were prepared weekly for the first three weeks of the study, and

monthly thereafter. Fresh batches of diet were provided to the animals on a weekly basis. Admixed diet was stored in the freezer until provided to animals at the start of the relevant week. The homogeneity and 28 day stability of prepared dietary admixtures were confirmed by HPLC using an internal standard (Batch No. 2009-000239, expiry 16 Jan 2011). Concentrations of test substance in diet were halved during the lactation period, to account for increased food consumption from lactating females.

Achieved mean concentrations as measured over the first 14 weeks of the study (P_1 pre-mating and gestation periods) were 100±1.9%, 99±3.2%, and 100±3.7% of nominal concentrations at 100, 500, and 1800 ppm, respectively (data excerpted from page 499 of the study report). In the P_1 lactation period, where nominal concentrations were reduced to 50, 250, and 900 ppm, achieved concentrations were 99±2.4%, 99±1.8%, and 100±3.3% of nominal concentrations, respectively.

Homogeneity analysis of concentrations from three samples each in the top, middle, and bottom layers of prepared batches (nine samples total/batch) at 20 ppm and 2500 ppm found achieved concentrations of 95±1.83% and 98±1.6% of target concentrations, respectively. Stability analyses of admixed diets at 20 and 2500 ppm after storage for seven days at room temperature indicated no decrease in test substance concentrations, with 99–105% of nominal concentration recovered. Stability analyses of frozen diet admixed with test substance reported similar results, with 96–101% of nominal concentration recovered from samples stored at 28 days at ≤ -18°C.

4. Statistics

Statistical methods employed in this study are listed in Table KIIA 5.6.2/02-4. Statistical significance was assessed to the $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ levels. Gross necropsy findings were not subjected to statistical analysis.. Historical control data from reproduction toxicity studies in Wistar rat performed by the laboratory over 1998 to 2008 were provided to assist in interpreting results.

Table KIIA 5.6.2/02-4. Statistics used in data analyses

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant (p>0.05)	**If preliminary test is significant (p≤0.05)**
Terminal bodyweight Organ weights	**Bartlett Test**	**Analysis of variance** followed by 2-sided **Dunnett's test if significant**	**Kruskal-Wallis test** followed by 2-sided **pairwise Mann-Whitney U Test if significant**
Parametric data, *e.g.*: Bodyweights Food consumption	**One-way analysis of variance** followed by 2-sided **Dunnett's test if significant**		
Non parametric data *e.g.*: No. of oestrus cycles, litter size, No. of implantation sites	**Kruskal-Wallis test** followed by **Dunn's test if significant**		
Nonparametric dichotomous Data, e.g.: Fertility, gestation index	**Chi-Square Test,** followed by **Fisher's Exact Test** with the Bonferroni Adjustment if significant		
Sperm data	**One-way analysis of variance**		
Ovarian follicles and corpora lutea counts	**Student's t-Test**		
Histopathology data	**Chi-Square test** followed by a one-tailed **Fisher's Exact test if significant**		

C. METHOD

Parental animals

During the treatment period, cage-side observations for mortality were performed twice daily during weekdays and once daily during weekends and public holidays. Any instances of behavioural changes, signs of difficult or prolonged delivery, and overt toxicity were recorded. In the event of animals displaying abnormal clinical signs, detailed physical assessment of animals outside of the cage was conducted as considered necessary.

Clinical observations, including detailed physical assessment were undertaken weekly throughout the study. Body weights and food consumption were recorded weekly during the pre-mating phase, with the exception of food consumption data, which was measured twice weekly during the first week of the pre-mating period. During gestation, dam body weight and food consumption were measured on GD 0, 6, 13 and 20. During lactation, dam body weight and food consumption were measured in LD 0, 4, 7, 14 and 21. Food use efficiency estimates were not reported. Fresh food was provided weekly.

Over a three-week period prior to mating, the estrous cycle was characterised for all P_1 and P_2-generation females, and was determined again just prior to termination.

During mating, one female and one male were co-housed for up to 14 days. Females showing positive signs of mating (by vaginal smear) were removed and housed individually thereafter during the gestation period. Parental males were removed from the study after litters were born, sacrificed by carbon dioxide asphyxiation, and subjected to necropsy and histopathological examination as required.

For all P_1- and P_2-generation males at termination, sperm was collected from one testis and one epididymis for enumeration of homogenisation-resistant spermatids and cauda epididymal sperm reserves, respectively. Sperm motility analysis for all male groups and sperm morphology analysis for control and high dose males of both generations was performed on sperm sampled from the distal portion (closest to the urethra) of the vas deferens. Epididymal counts were recorded for control and high dose P_1-generation males, and at all dietary levels for P_2-generation males.

Females that did not litter (with or without sperm-positive smears and/or vaginal plugs) were sacrificed by carbon dioxide asphyxiation and subjected to necropsy after GD24 (or 24 days after the end of the mating period). For these animals, patency of the cervical/uterine ostium in these females was examined by flushing uterine horns with 10% buffered formalin. Maternal animals producing litters were sacrificed after the weaning of litters on LD21 and necropsied. For all parental animals, terminal measurements (body weights, organ weights, gross pathology and histopathology) were conducted as outlined in Table KIIA 5.6.1/02-5 below. The uterus from maternal animals was also excised and counted for implantations.

A quantitative evaluation of the ovarian follicles (preantral and antral) and corpora lutea was conducted on the first 10/dose F_1-dams that delivered pups from the control and high dose groups (20 dams in total). For each dam, follicles in both ovaries were counted, using five step sections per ovary.

In general, histopathological analysis was undertaken on control and high dose groups only, with

the exception of reproductive tissues of any animals suspected of reduced fertility or impaired sperm motility. In the event of abnormal findings, tissue from lower dose groups was analysed to establish test substance relationships until a no-observed adverse effect level (NOAEL) was identified.

Table KIIA 5.6.1/02-5: Organs/tissues collected from adult P_1 and P_2 animals for histopathological examination and reproductive status evaluation ('X' – denotes that the procedure was undertaken)

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted[a]
Adrenal gland	X	X	X
Brain	X	X	-
Coagulating gland	-	X	X
Epididymis	X	X	X
Epididymis cauda (side not utilised for sperm)	X	X	X
Kidney	X	X	X
Liver	X	X	X
Lung	-	X	X
Ovary	X	X	X
Physical identifier	-	X	-
Pituitary	X	X	X
Prostate	X	X	X
Seminal vesicle (with coagulating gland and fluid)	X	X	X
Spleen	X	X	X
Testis	X	X	X
Thymus	X	X	-
Thyroid gland	X	X	-
Uterus with oviduct and cervix	X	X	X
Vagina	-	X	X
Gross lesions	-	X	X

Histopathological analysis was undertaken on control and high dose groups only, with the exception of reproductive tissues of animals with impaired fertility. In the event of abnormal findings in the high dose group, relevant samples from the mid dose group were subjected to histopathological analysis. Data excerpted from page 27 of the study report.

Offspring animals

F_1 and F_2 litters were culled to 4 pups/sex/litter (sex and litter size permitting) on postnatal day (PND) 4; all remaining pups were sacrificed by decapitation, with grossly abnormal pups evaluated macroscopically. A summary of offspring-related parameters examined across both generations is shown in Table KIIA 5.6.1/02-6 below.

Table KIIA 5.6.1/02-6: Litter parameters

Observation	Time of Observation (Lactation Day)					
	0	**4**	**7**	**14**	**21**	**0-21**
Number of Live Pups						**X**
Pup Weight	**X**	**X**	**X**	**X**	**X**	
Clinical Observations						**X**
Number of Dead Pups						**X**
Sex of each Pup (M/F)	**X**					

Preputial Separation[a]	Performed post weaning
Vaginal Patency[a]	Performed post weaning
Anogenital Distance[b]	Measured PND 0

'a' – Selected F1 pups only. 'b' – F2 pups only. Data excerpted from page 28 of the study report.

At PND21, animals from the F_1 litters not selected as P_2 parents, and all F_2 animals were sacrificed by CO_2 asphyxiation. One pup/sex/litter for both F_1 and F_2 generations were chosen at random for necropsy, tissue collection, organ weight determination and histopathological analysis, focussing on structural abnormalities or pathological changes and those in the reproductive system in particular (see Table KIIA 5.6.1/02-7 below). Stillborn and prematurely dead pups in both generations were subjected to gross necropsy to determine the cause of death.

Table KIIA 5.6.1/02-7: Organs/tissues collected from 1/sex/litter pups for histopathological examination ('X' – denotes that the procedure was undertaken)

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted[a]
Brain	X	X	-
Cervix	-	X	X
Coagulating gland	-	X	X
Epididymis	-	X	X
Ovary	-	X	X
Oviduct	-	X	X
Prostate	-	X	X
Seminal vesicle	-	X	X
Spleen	X	X	-
Testis	-	X	X
Thymus	X	X	-
Uterus	X	X	X
Vagina	-	X	X
Gross lesions	-	X	X

Histopathological analysis was only undertaken on 1 pup/sex/litter for each generation. Data excerpted from page 28 of the study report.

II. RESULTS AND DISCUSSION

A. PARENTAL TOXICITY (P_1 and P_2 rats)

1. Clinical observations

There was no test substance related effect on mortality or clinical signs of toxicity.

2. Bodyweight, bodyweight gain, and food consumption

Pre-mating (10 weeks) phase

Bodyweight and bodyweight gain

For P_1 males during the 10-week premating study phase, bodyweights and bodyweight gain were unaffected by the test substance at all dose levels.

For P_1 females, bodyweights and bodyweight gains were unaffected by treatment at 100

ppm. At 500 ppm, bodyweight gains over the 10-week premating period were 21% lower than those of controls, resulting in final pre-mating bodyweights being 4% lower than controls (Table KIIA 5.6.1/02-8). Noting the very slight decrease in final pre-mating bodyweight, the decreased bodyweight gain is treatment-related but unlikely to be toxicologically adverse. At 1800 ppm, an overall 43% decrease in mean bodyweight gain was observed, resulting in decreased mean bodyweight (-10%; $p \leq 0.01$ vs controls) by the end of the pre-mating period.

For P_2 males, bodyweights and bodyweight gain at 100 ppm were unaffected by treatment during the pre-mating period. While 500 ppm males started the pre-treatment period with decreased body weight compared with controls (-5%, not statistically significant;), the mean bodyweight gain observed during the pre-mating period was slightly greater than controls, resulting in a final pre-mating bodyweight comparable to controls. P_2 males at 1800 ppm started the pre-mating period with 12% lower bodyweights compared with controls, and mean bodyweights remained lower than controls by a similar magnitude throughout the pre-mating period. The mean bodyweight gain during the pre-mating phase was slightly decreased compared with controls, but % bodyweight gain (as a percentage of the initial body weight at the beginning of the pre-mating phase) was comparable among all dose groups. Therefore, the bodyweight effects noted at 1800 ppm in P_2 males are a function of the delayed growth during the pre-weaning period and are not a direct effect of treatment during the pre-mating phase.

For P_2 females at 100 ppm, bodyweight and bodyweight gains were unaffected over the 10-week premating phase. At 500 ppm and 1800 ppm, initial pre-mating body weights were decreased compared with controls (-5% and -15%; $p<0.05$ and $p<0.01$ respectively;), and decreased absolute bodyweight gain was observed at both dose levels (-16 to -21%) during the pre-mating period. However, as with the P_2 males, the magnitude of the bodyweight decrease from controls remained constant over the pre-mating period, and relative bodyweight gains were comparable among all dose groups. (Table KIIA 5.6.1/02-8). As such, the bodyweight effects noted at 1800 ppm in P_2 females are considered to reflect the delayed growth during the pre-weaning period and are not a direct effect of treatment during the pre-mating phase.

Overall, the decreased bodyweights in P_2 males and females reflected the reduced growth during the pre-weaning period, and were not considered to be related to pre-mating exposures. Only the decreased bodyweight gain observed in P_1 females at 1800 ppm and the decreased bodyweight observed in P_1 females at 1800 ppm were considered treatment-related and adverse effects on parental animals.

Food consumption

Food consumption was unaffected by treatment in P_1 males. A slightly increased food consumption compared to controls was observed in ≥500 ppm P_2 males when expressed as a g/kg/day measure (+4%/+11% for 500/1800 ppm respectively); however, food consumption on a per animal basis was similar to controls.

In P_1 females, a statistically significant decrease (-10%) in mean food consumption (expressed as g/animal/day) was observed at 1800 ppm, though food consumption expressed as g/kg/day was similar to controls. The decreased food consumption is likely to have partially contributed to the decreased mean bodyweight gain (-43% vs controls) observed at the same dose level. The decreased mean food consumption of -12% (g/animal/day) in P_2

females at 1800 ppm likely reflected the smaller size of these animals.

While no notable changes in food consumption were noted in 500 ppm P_1 females, the decreased mean bodyweight gains observed may suggest decreased food use efficiency in these animals.

Table KIIA 5.6.1/02-8: Bodyweight and food consumption over 10 week pre-mating phase

Study week	Dose (ppm)			
	0	100	500	1800
P_1-Generation males- pre-mating				
Mean bw (g) – Initial±S.E.	235.2±3.30	234.1±3.04	241.3±3.49	240.4±3.19
Mean bw (g) - Wk 14 ±S.E.	436.0±7.81	436.0±5.96	441.3±7.77	438.4±7.54
Mean bw gain (g) Wks 1–14	200.8	201.9	200.0	198.0
% bw gain (from initial bw)	85.4	86.2	82.9	82.4
Mean food cons. (g/animal/day) Wks 1–10	21.4	21.9	21.9	21.7
Mean food cons. (g/kg/day) Wks 1–10	65.4	67.2	65.6	65.5
P_1-Generation females - pre-mating				
Mean bw (g) – Initial±S.E.	174.0±2.52	173.7±2.09	178.6±2.27	177.0±1.84
Mean bw (g) - Wk 10 ±S.E.	237.4±3.33	235.5±3.09	229.0±2.92 (-4%)	213.3±2.73** (-10%)
Mean bw gain (g) Wks 1–10	63.4	61.8	50.4 (-21%)	36.3 (-43%)
% bw gain (from initial bw)	36.4	35.6	28.2 (-23%)	20.5 (-44%)
Mean food cons. (g/animal/day) Wks 1–10	16.3	16.0	16.0	14.7** (-10%)
Mean food cons. (g/kg/day) Wks 1–10	78.5	77.6	78.1	76.4
P_2-Generation males- pre-mating				
Mean bw (g) – Initial±S.E.	302.2±5.06	304.0±4.94	286.1±5.15 (-5%)	265.7±5.10** (-12%)
Mean bw (g) - Wk 14 ±S.E.	454.5±8.27	456.0±7.51	452.3±8.28	410.8±7.53** (-10%)
Mean bw gain (g) Wks 1–14	152.3	152.0	166.2 (+9%)	145.1 (-5%)
% bw gain (from initial bw)	50.4	50.0	58.1	54.6
Mean food cons. (g/animal/day) Wks 1–10	23.4	23.8	23.4	22.8
Mean food cons. (g/kg/day) Wks 1–10	62.2	63.7	64.5 (+4%)	68.8** (+11%)
P_2-Generation females - pre-mating				
Mean bw (g) – Initial±S.E.	189.9±2.77	185.9±2.78	180.5±2.30* (-5%)	162.3±2.34** (-15%)
Mean bw (g) - Wk 10 ±S.E.	242.1±3.04	237.3±4.06	224.2±2.90** (-7%)	203.5±2.49** (-16%)
Mean bw gain (g) Wks 1–10	52.2	51.5	43.7 (-16%)	41.2 (-21%)
% bw gain (from initial bw)	27.5	27.7	24.2	25.3
Mean food cons. (g/animal/day) Wks 1–10	16.8	16.3	16.3	14.8** (-12%)
Mean food cons. (g/kg/day) Wks 1–10	77.7	76.8	79.8 (+3%)	80.7 (+4%)

** - denotes statistically significant at the $p \leq 0.01$ level. The study author only provided standard error data for the mean bodyweight parameter. Data excerpted from pages 31, 49, 51, 53 and 55 of the study report, with the exception of per cent change from control rounded to integers as well as the percent bw gain from initial bw, which were calculated by the evaluator using Microsoft Excel.

Gestation phase (GD 0 to 20)

There were no instances of group mean bodyweight losses over the weekly gestation intervals during gestation in the P_1 or P_2 generations. For both P_1 generation and P_2 generation dams during gestation, bodyweight gains, mean bodyweights and food

consumption were unaffected at 100 or 500 ppm (Table KIIA 5.6.1/02-9). While a decreased mean bodyweight was noted in P_2 females at 500 ppm during the gestation period, this was considered a carry-over effect from the pre-mating period, and noting the similar bodyweight gain and food consumption compared with controls, the bodyweight was considered to be unaffected by treatment at 500 ppm.

At 1800 ppm, decreased bodyweights compared with controls were observed at GD0 in both generations, and were considered carry-over effects from the pre-mating period. In both generations, decreased bodyweight gain compared with controls was observed (statistically significant in P_2), but % bodyweight gains relative to GD 0 were comparable among all dose groups. However, relative food consumption was increased compared with controls, particularly in P_2 females, suggesting a pattern of treatment-related decreased food use efficiency.

Table KIIA 5.6.1/02-9: Bodyweight gain and food consumption during gestation

Study week	Dose (ppm)			
	0	100	500	1800
P_1-Generation females				
$\bar{x}$ bw (g) - GD 0	238.1±4.27	235.2±3.25	232.2±3.10	214.8±3.06** (-10%)
$\bar{x}$ bw (g) - GD 6	255.0±3.65	250.2±3.07	248.6±3.22	228.1±3.25** (-11%)
$\bar{x}$ bw (g) – GD 13.	276.0±3.54	272.3±3.57	267.9±3.63	247.1±3.06** (-10%)
$\bar{x}$ bw (g) - GD 20	333.6±5.68	326.5±4.28	322.1±5.41	301.1±4.43** (-10%)
$\bar{x}$ bw gain (g) GD 0–20	95.6±3.63	91.3±2.59	89.9±3.74	86.4±2.87 (-10%)
% bw gain (from GD 0 bw)	40.2	38.8	38.7	40.2
$\bar{x}$ food cons. (g/animal/day) GD 0–20	18.1	17.9	17.9	17.5
$\bar{x}$ food cons. (g/kg/day) GD 0–20	70.6	70.9	71.6	76.2 (+8%)
P_2-Generation females				
$\bar{x}$ bw (g) - GD 0	243.4±3.27	238.3±4.82	224.4±3.01** (-8%)	202.6±2.85** (-17%)
$\bar{x}$ bw (g) - GD 6	257.6±3.09	251.0±4.57	238.7±2.86** (-7%)	214.0±3.23** (-17%)
$\bar{x}$ bw (g) – GD 13.	277.8±3.33	270.0±4.54	258.3±2.80** (-7%)	230.8±2.98** (-17%)
$\bar{x}$ bw (g) - GD 20	335.1±4.52	327.4±5.28	313.8±4.01** (-6%)	277.2±4.28** (-17%)
$\bar{x}$ bw gain (g) GD 0–20	91.8±2.59	89.1±3.50	89.4±2.86	74.7±2.11** (-19%)
% bw gain (from GD 0 bw)	37.7	37.4	39.8	36.9
$\bar{x}$ food cons. (g/animal/day) GD 0–20	18.2	17.6	17.8	19.0
$\bar{x}$ food cons. (g/kg/day) GD 0–20	70.2	69.7	74.3 (+6%)	89.3** (+27%)

** - denotes statistically significant at the p≤0.01 level. The study author did not provide standard error data for food consumption values. Data excerpted from pages 32 and 33 of the study report, with the exception of per cent change from control rounded to integers as well as the percent bw gain from initial bw, which were calculated by the evaluator using Microsoft Excel.

Lactation phase (LD 0 to 21)

For P_1 generation dams during lactation days (LD) 0 to 21, bodyweight gains and bodyweights at 100 or 500 ppm were unaffected by treatment (Table KIIA 5.6.1/02-10). At

1800 ppm, starting bodyweights at LD 0 were decreased by 11% compared with controls ($p \leq 0.01$), with a marginal recovery in bodyweights to an 8% decrease compared with controls ($p \leq 0.01$) by LD 21, noting a 7% increase in mean food consumption (g/kg/day) compared with controls and a 35% increased bodyweight gain over the lactation period.

For P_2 generation dams during LD 0 to 21, bodyweights and bodyweight gains at 100 ppm were unaffected by dosing. At 500 ppm, net bodyweight gain and mean food consumption were similar to controls, with initial and final bodyweights proportionally unchanged (-8%) compared with controls. At 1800 ppm, starting bodyweights at LD 0 were 17% ($p \leq 0.01$) lower than controls, with a marginal recovery in bodyweights to a 13% decrease compared with controls ($p \leq 0.01$) by LD 21, noting a 7% increase in mean food consumption (g/kg/day) compared with controls and a 38% increased bodyweight gain over the lactation period.

Table KIIA 5.6.1/02-10: Bodyweight gain and food consumption during lactation

Study week	Dose (ppm)			
	0	100	500	1800
P_1-Generation females				
$\bar{x}$ bw (g) - LD 0	262.8±3.22	256.1±2.95	250.8±4.30	233.3±3.24** (-11%)
$\bar{x}$ bw (g) - LD 4	271.3±3.12	271.6±3.36	265.8±3.91	245.7±3.66** (-9%)
$\bar{x}$ bw (g) – LD 7	277.4±2.81	276.7±3.05	271.2±3.90	251.6±3.20** (-9%)
$\bar{x}$ bw (g) – LD 14	292.6±3.81	292.3±2.77	287.2±4.22	267.1±3.17** (-9%)
$\bar{x}$ bw (g) – LD 21	285.8±3.67	281.4±2.80	280.1±3.59	264.3±3.15** (-8%)
$\bar{x}$ bw gain (g) LD 0–21	23.0	25.3	29.3 (+27%)	31.0 (+35%)
% bw gain (from LD 0 bw)	8.8	9.9	11.7	13.3
$\bar{x}$ food cons. (g/animal/day) LD 0–21	41.5	43.1	41.6	40.2
$\bar{x}$ food cons. (g/kg/day) LD 0–21	149.2	155.9	153.3	159.8 (+7%)
P_2-Generation females				
$\bar{x}$ bw (g) - LD 0	263.0±3.38	254.5±4.53	242.5±3.27** (-8%)	218.4±2.84** (-17%)
$\bar{x}$ bw (g) - LD 4	277.3±3.24	265.9±4.13	256.0±3.51** (-8%)	225.1±3.69** (-19%)
$\bar{x}$ bw (g) – LD 7	278.7±3.23	268.5±4.02	260.9±3.43** (-6%)	231.3±3.02** (-17%)
$\bar{x}$ bw (g) – LD 14.	294.8±3.54	282.8±3.64	272.2±3.72** (-8%)	244.3±3.53** (-17%)
$\bar{x}$ bw (g) – LD 21	283.8±3.45	278.5±3.91	261.6±4.17** (-8%)	247.1±3.22** (-13%)
$\bar{x}$ bw gain (g) LD 0–21	20.8	24.0	19.1	28.7 (+38%)
% bw gain (from LD 0 bw)	7.9	9.4	7.9	13.1
$\bar{x}$ food cons. (g/animal/day) LD 0–21	45.3	42.5	44.0	40.1 (-11%)
$\bar{x}$ food cons. (g/kg/day) LD 0–21	162.0	158.5	169.4 (+5%)	173.2 (+7%)

** - denotes statistically significant at the $p \leq 0.01$ level. Data excerpted from page 34 of the study report, with the exception of per cent change from controls rounded to integers which were calculated by the evaluator using Microsoft Excel, and bodyweight gain data as well as the percent bw gain from initial bw, which were calculated manually.

3. Compound intakes

Achieved test substance intakes are presented in Table KIIA 5.6.1/02-2 in the study methods.

4. Reproductive parameters, sperm evaluation and oestrus cycling

No treatment-related effects on P_1 generation reproductive parameters were observed (Table KIIA 5.6.1/02-11). Sperm motility and morphology was unaffected by treatment at all dose levels, and estrus cycling patterns were similar to controls (Table KIIA 5.6.1/02-12). Testicular and epididymal sperm counts were slightly reduced (-12 to -13%; not statistically significant) in P_1 males at 1800 ppm; however these parameters were not assessed in the low and mid dose groups, making interpretation of these findings difficult. Nonetheless, the decrease in sperm counts was considered to be compound- related and adverse.

In P_2 parental animals, male reproductive parameters were unaffected by treatment, with the exception of a slight reduction in epididymal sperm count at 1800 ppm when compared to controls (-11%; not statistically significant). In females, an 8% ($p \leq 0.01$) decrease in the total number of implantation sites and a 14% decreased in the mean number of implantation sites at 1800 ppm were observed compared with controls (Table KIIA 5.6.1/02-11). Additionally, P_2 females at 1800 ppm had a 17% decrease ($p \leq 0.05$) in the number of estrus cycles relative to controls, though the overall oestrus cycle length was unaffected by treatment (Table KIIA 5.6.1/02-12). The study director has indicated that the decrease in number of oestrus cycles at 1800 ppm paralleled the decreased bodyweight gain observed in the same dose group: the evaluator considers the effect to be treatment-related.

Table KIIA 5.6.1/02-11: Parental reproductive indices in two-generation reproduction study

	Dose (ppm)			
	0	**100**	**500**	**1800**
P_1-Generation, F_1-Offspring				
No. Cohoused	30	30	30	30
No. Mated	30	29	29	30
No. Delivered	29	27	28	28
No. with Implants	29	27	28	28
Mating Index	100	96.7	96.7	100
Fertility Index	96.7	93.1	96.6	93.3
Gestation Index	100	100	100	100
$\bar{x}$ No. Days to Insemination	3.4±0.67	3.3±0.59	3.2±0.52	3.1±0.59
Median	3	3	3	3
$\bar{x}$ Gestation Length (days)	22.1±0.11	22.1±0.12	22.1±0.12	22.0±0.12
Median	22	22	22	22
Total No. of implants (Median)	311 (11.0)	285 (11.0)	298 (10.5)	289 (10.0)
Mean No. of implants (±S.E.)	10.7±0.63	10.6±0.52	10.6±0.57	10.3±0.47
P_2-Generation – F_2-Offspring				
No. Cohoused	30	30	30	30
No. Mated	29	30	29	30
No. Delivered	27	28	28	29
No. with Implants	27	28	28	29
Mating Index	96.7	100	96.7	100
Fertility Index	93.1	93.3	96.6	96.7
Gestation Index	100	100	100	100
$\bar{x}$ No. Days to Insemination	2.3± 0.23	3.7±0.45	2.6±0.24	2.1±0.18
Median	2	4.0**	2.5	2
$\bar{x}$ Gestation Length (days)	22.0± 0.09	22.1±0.15	21.9±0.09	22.0±0.08
Median	22	22	22	22
Total No. of implants (Median)	305 (12.0)	314 (11.0)	323 (11.0)	281 (10.0)** [-8%]
Mean No. of implants (±S.E.)	11.3±0.50	11.2±0.49	11.5±0.32	9.7±0.33 [-14%]

**- denotes statistically significant at the $p \leq 0.01$ level. Statistical significant findings were confined to the P_2 generation. Mating index = No. mated/No. cohabited. Fertility index = No. pregnant/No. mated. Gestation index = No. with liveborn/No. with evidence of pregnancy. Data excerpted from pages 37, 69 and 70 of the study report, except for [per cent change from control] rounded to integers, which was calculated by the evaluator using Microsoft Excel.

Table KIIA 5.6.1/02-12. Summary data on sperm parameters and oestrous cycle

	Dose (ppm)			
	0	**100**	**500**	**1800**
Sperm parameter data				
P_1 generation:				
Motility				
% motile	92.4±4.3	90.9±12.4	93.3±3.3	90.7±5.3
% progressive	62.1±8.5	60.7±12.6	63.3±6.5	59.2±7.3
Counts				
testis (10^6 sperm/g)	26.9±8.6	N/A	N/A	23.7±5.5 [-12%]
epidid.(10^6 sperm/g)	158.8±65.6	N/A	N/A	138.7±56.4 [-13%]
Morphology (# in 200 total sperm)				
normal (mean total No.)	193.2±20.5	N/A	N/A	197±1.9
abnormal (mean total No.)	2.7±2.1	N/A	N/A	2.2±1.7
detached head (mean total No.)	4.1±20.0[a]	N/A	N/A	0.8±1.1
P_2 generation:				
Motility				
% motile	90.7±16.4	94.5±2.5	92±13.3	92.7±4.9
% progressive	64±13.3	66.4±4.8	64.8±11.5	63.3±6.7
Counts				
testis (10^6 sperm/g)	29.4±8.5	N/A	N/A	30.9±8.4
epidid.(10^6 sperm/g)	158.8±58.7	N/A	N/A	141.8±36.0 [-11%]
Morphology (# in 200 total sperm)				
normal (mean total No.)	189.1±35.9	N/A	N/A	195.2±2.9
abnormal (mean total No.)	3.8±3.3	N/A	N/A	4.2±2.6
detached head	0.4±0.9	N/A	N/A	0.6±1.0
Oestrous parameter data				
P_1 generation				
No. of estrous cycles ±S.E.	3.5±0.1	3.7±0.1	3.4±0.2	3.4±0.1
Estrous cycle length ±S.E.	4.4±0.3	4.3±0.1	4.3±0.2	4.3±0.1
F_1 generation				
No. of estrous cycles ±S.E.	3.5±0.2	3.3±0.2	3.3±0.2	2.9±0.2* (-17%)
Estrous cycle length ±S.E.	4.0±0.2	4.1±0.1	4.4±0.2	4.4±0.1

*- denotes statistically significant at the p≤0.05 level. Statistical significant findings were confined to P_2 generation females. Data excerpted from pages 35 and 36 of the study report, with the exception of per cent change from controls rounded to integers which were calculated by the evaluator using Microsoft Excel. Standard deviations for the sperm analysis results were also calculated by the PMRA evaluator using Microsoft Excel. [a] One control P1 male (rat number SA0005) had a value for detached head of 110; the group mean±S.D. when this value is excluded becomes 0.48±0.78.

5. Gross pathology, organ weights, and histopathology in parental animals

Gross necropsy findings at scheduled sacrifice for both parental and offspring animals were unaffected by treatment.

In P_1 organ weight analyses, treatment-related organ weight changes were identified in 1800

ppm males, with statistically significant changes in absolute (+9%) and relative-to-bodyweight (+9%) liver weights, and absolute (+17%) and relative-to-bodyweight (+33%) thyroid weights (Table KIIA 5.6.1/02-13). The increased liver weights were considered to be associated with hypertrophy observed in histopathology (see below). No histopathological correlate was identified for the increased thyroid weights. A statistically significant increase in absolute and relative thyroid weights observed in 500 ppm females was not considered treatment-related due to the lack of dose relationship and histopathological changes at the top dose of 1800 ppm (which did not present with treatment-related thyroid weight changes).

In P_1 females, statistically significant ($p<0.05$) decreases in absolute spleen (-14%) and thymus (-15%) were noted at 1800 ppm (data not shown). No changes in relative organ weights or corresponding pathological lesions were noted, and these decreases are likely related to the decreased body weight noted in these animals.

In P_2 organ weight analyses, treatment-related absolute organ weight changes were observed in female thyroids (-14%, combined weight, not statistically significant) at 1800 ppm; however, the relative-to-bodyweight value was similar to controls. Additionally, statistically significant increases in the relative-to-bodyweight liver weight were observed in both sexes at 1800 ppm (+9%/+11% for M/F respectively); however, as no treatment-related liver histopathological changes were identified in P_2 animals at 1800 ppm, it is unlikely the observed small increase in relative liver weight is toxicologically adverse.

In P_2 males, statistically significant ($p<0.5$) decreases in absolute brain (-4%), thymus (-15%), kidney (-8 to -9%), testes (-8%) and epididymal (-8 to -15%) weights were noted at 1800 ppm (data not shown). No changes in relative organ weights or corresponding pathological lesions were noted, and these decreases are likely related to the decreased body weight noted in these animals.

Statistically significant ($p<0.05$) decreases in absolute epididymal weights (-7 to -10%) were also noted in P2 males at 500 ppm (data not shown). No changes in relative organ weights or corresponding pathological lesions were noted, and these decreases are likely related to the decreased body weight noted in these animals.

In P_2 females, statistically significant decreases in absolute brain (-5%), spleen (-15%), pituitary (-15%), adrenal gland (-12 to -19%), kidney (-12-13%), and ovary (right only; -16%) weights were observed at 1800 ppm (data not shown). Relative-to-bodyweight brain weight was statistically significantly elevated (+8%; $p<0.05$) when compared to controls (data not shown), but no changes in relative weights for other organs were observed, nor were there any corresponding pathological lesions, thus these changes are likely related to the decreased body weight noted in these animals.

Table KIIA 5.6.1/02-13: Parental organ weight data

	ppm [N=30]			
	0	**100**	**500**	**1800**
Terminal bw (g)				
P_1 males	442.9±44.8	443.9±33.7	445.3±42.7	442.4±43.7
P_1 females	287.1±20.0	280.4±16.9	279.1±21.6	264.8±20.1* (-8%)
P_2 males	462.2±52.8	466.9±43.9	459.4±44.5	416.0±42.1* (-10%)
P_2 females	281.6±21.5	279.3±21.5	264.7±22.2* (-6%)	247.5±18.9* (-12%)
Liver weights				
P_1 males				
Abs weight (g)	15.153±1.642	14.944±1.859	15.219±2.013	16.564±1.736* (+9%)
Rel to bw (%)	3.424±0.188	3.360±0.246	3.419±0.333	3.749±0.240* (+9%)
P_1 females				
Abs weight (g)	12.968±1.377	12.365±1.617	12.463±1.867	12.558±1.602
Rel to bw (%)	4.514±0.318	4.397±0.422	4.455±0.507	4.732±0.411
P_2 males				
Abs weight (g)	15.603±2.652	16.081±1.959	15.923±1.552	15.230±1.654
Rel to bw (%)	3.360±0.318	3.441±0.218	3.472±0.202	3.666±0.248* (+9%)
P_2 females				
Abs weight (g)	12.830±1.502	12.919±1.695	12.708±1.527	12.580±1.634
Rel to bw (%)	4.552±0.374	4.622±0.464	4.799±0.406	5.072±0.438* (+11%)
Thyroid weights (shown as combined left and right lobes due to similar values)				
P_1 males				
Abs weight (g)	0.029±0.004	0.031±0.005	0.030±0.012	0.034±0.004* (+17%)
Rel to bw	0.006±0.001	0.007±0.001	0.006±0.001	0.008±0.001* (+33%)
P_1 females				
Abs weight (g)	0.020±0.003	0.020±0.004	0.023±0.004* (+15%)[A]	0.021±0.003
Rel to bw	0.007±0.001	0.008±0.001	0.008±0.001* (+14%)[A]	0.008±0.001
P_2 males				
Abs weight (g)	0.029±0.006	0.032±0.006	0.032±0.006	0.032±0.006
Rel to bw	0.006±0.001	0.007±0.001	0.007±0.001	0.008±0.001* (+33%)
P_2 females				
Abs weight (g)	0.022±0.004	0.020±0.004	0.021±0.004	0.019±0.004 (-14%)
Rel to bw	0.008±0.001	0.008±0.001	0.008±0.001	0.008±0.001

Note: Standard deviations for the thyroid are presented as the mean standard deviation from the left and right lobes and rounded to three decimal places. [A] – statistically significant for the one thyroid lobe only. Standard deviations for the left and right lobe were calculated by the formula: SDt = Sqrt(SD_L^2+ SD_R^2). Data excerpted from pages 651 to 673 of the study report, with the exception of per cent change from controls rounded to an integer, which were calculated by the evaluator using Microsoft Excel.

Treatment-related histopathological findings for parental adults across both generations were confined to a 27% incidence of centrilobular hepatocellular hypertrophy of a minimal severity in P_1 generation adult males at 1800 ppm (versus a nil finding in any other group of the P_1 or P_2 generations: Table KIIA 5.6.1/02-14). The pathologists' report described the lesions as: "…enlarged hepatocytes primarily involving the cytoplasm of centrilobular areas. The cytoplasmic appearance of hepatocytes varied from granular or intensively eosinophilic or pale". The study authors regarded this lesion to be an adaptive response to xenobiotic load, and that it correlated to the increased liver weights in P_1 generation males at this dose. The evaluator agrees with both conclusions.

Table KIIA 5.6.1/02-14: Incidences of hepatocellular hypertrophy (centrilobular) of minimal severity in adults

	ppm			
	0	**100**	**500**	**1800**
Males:				
P_1 Generation adults (% incidence)	0/30	0/1	0/29	8/30* (27%)
P_2 Generation adults	0/30	0/0	0/0	0/30
Females:				
P_1 Generation adults	0/30	0/0	0/0	0/30
P_2 Generation adults	0/30	0/0	0/0	0/30

*Denotes statistical significance at the $p \leq 0.05$ level. Data excerpted from pages 685 to 691 of the study report, with the exception of per cent incidence which was calculated by the evaluator and rounded to an integer.

Ovarian follicle counts for P_2 generation females at 1800 ppm were unremarkable, with mean preantral follicles (7.81 for controls vs 7.52, $p = 0.67$), mean antral follicles (4.14 for controls vs 4.48, $p = 0.52$), and corpora lutea (4.36 for controls vs 3.87, $p = 0.15$) values similar to untreated controls. Ovarian follicle counts for P_1 generation females were not provided.

B. F_1 AND F_2 LITTER AND PUP DATA

1. General observations

   Clinical signs in F_1 and F_2 pups were confined to mild dehydration in F_1 pups from the 100 and 500 ppm dose groups where water was accidentally not provided for a 24 hour period. No clinical signs of toxicity associated with test substance administration were observed.

2. Litter size and pup survival

   Findings on litter size and pup survival parameters were confined to a 15% decrease in litter size (9.2 versus 10.8) in F_2-generation pups at 1800 ppm relative to controls (Table KIIA 5.6.1/02-15). The study authors correlated this finding to the 19% ($p \leq 0.01$) lower GD 0 to 20 bodyweight gain and 8% lower ($p \leq 0.01$) number of implantations in P_2 dams relative to controls, and also noted that reported mean litter size at 1800 ppm was just below the historical control range in the laboratory (9.8 to 11.8). This decrease was slight and was not considered as adverse.

   Additionally, a non-statistically significant 9% decrease ($p \leq 0.05$ for median) in the total number of pups born relative to controls was seen in P_2 dams at 1800 ppm, though this is considered an effect associated with the decreased number of implantations observed.

   A decrease in the per cent of males born was seen at 1800 ppm in both the P (49.6% vs 55.1% males in controls) and F1 generation (47.7% vs 51.6% males in controls), but these findings were without statistical significance or dose dependency, and both ratios in the test

substance groups were very close to 50%. The study authors did not specifically discuss the finding, but the evaluator considers that sex ratios were unaffected by test substance administration.

There were no effects on stillbirth incidence, birth, live birth, viability and lactation indices, or other parameters of pup survival. Although there were slightly more stillborn pups at 1800 ppm in the F_2 generation than in lower dose groups, the incidence was low (no historical control data were provided for comparison). There was also a slightly increased incidence of pup losses after birth at 1800 ppm in the F_2 generation when compared to lower dose groups, but the incidence was comparable to the control value in the F_1 generation (no historical control data were provided for comparison).

Table KIIA 5.6.1/02-15: Litter size and pup survival for F_1 and F_2 generations

	Dose (ppm)			
	0	**100**	**500**	**1800**
F_1 GENERATION				
No. of litters with liveborn pups	29	27	28	28
Total No. of pups born	297	277	282	281
No. stillborn	0	2 (2 litters)	0	0
Total No. of liveborn pups	297	275	282	281
Sex Ratio Day 0 (%M)	55.1	53	54.7	49.6
Mean litter size (day 0)	10.2±0.65	10.3±0.51	10.1±0.62	10±0.47
Median litter size (day 0)	11	11	10	10
Total no. of pups missing (no. of litters)	7 (3)	3 (3)	1 (1)	7 (5)
Total no. of pups found dead (no. of litters)	2 (2)	0 (0)	0 (0)	1 (1)
Pup losses PND 0-4 (no. of litters)[#]	7 (3)	2 (2)	1 (1)	6 (6)
Pup losses PND 5-21 (no. of litters)[#]	2 (2)	1 (1)	0 (0)	2 (2)
Total pup losses PND 0-21 (no. of litters)[#]	9 (4)	3 (3)	1 (1)	8 (6)
Birth index	95	97.1	93.3	97.4
Live birth index	100	99	100	100
Viability index	98	99.3	99.7	98.1
Lactation index	99.1	99.5	100	99.1
F_2 GENERATION				
No. of litters with liveborn pups	27	28	28	29
Total No. of pups born	291	300	311	266 (-9%)
No. stillborn	1	0	1	4 (3 litters)
Total No. of liveborn pups	290	299	309	262
Sex Ratio Day 0 (%M)	51.6	50.6	50.6	47.7
Mean litter size (day 0)	10.8±0.49	10.7±0.58	11.1±0.33	9.2±0.35 (-15%)
Median litter size (day 0)	11	11	10.5	10* (-9%)
Total no. of pups missing (no. of litters)	1 (1)	0 (0)	1 (1)	6 (3)
Total no. of pups found dead (no. of litters)	3 (2)	1 (1)	1 (1)	2 (2)
Pup losses PND 0-4 (no. of litters)[a]	2 (1)	1 (1)	2 (2)	6 (4)
Pup losses PND 5-21 (no. of litters)[a]	2 (2)	0 (0)	0 (0)	2 (2)
Total pup losses PND 0-21 (no. of litters)[a]	4 (3)	1 (1)	2 (2)	8 (5)
Birth index	95.6	92.7	96.4	94.8
Live birth index	99.7	99.7	99.2	98.2
Viability index	99.3	99.7	99.4	97.2
Lactation index	99.1	100	100	98.7

* - - denotes statistically significant at $p \leq 0.05$ level. Data excerpted from pages 39, 91-92 and 105-108 of the study report, with the exception of per cent change from control, which was calculated by the evaluator using Microsoft Excel. [a] pup losses were calculated by the evaluator from the individual litter data on pages 331-334 and 339-342 of the study report.

3. Pup body weight

Birth weights and bodyweight gains during the weaning period were unaffected at 100 ppm in both F_1 and F_2 generations (Table KIIA 5.6.1/02-16).

At 500 ppm, a slight decrease in F_1 mean bodyweights (-3to -5% for males, -3 to -6% for females) and bodyweight gain (-4% for males, -5% for females) during the weaning period were observed. Noting that decreases in bodyweight and bodyweight gain in the 500 ppm F_1 litters were minor and without statistical significance during the weaning period, it is unlikely the observed changes are toxicologically adverse in this case. However, in F_2 litters, statistically significant decreases in mean bodyweight (-6 to -7% vs controls; $p \leq 0.05$) during the latter portion of the weaning period (LD 14–21) and overall bodyweight gain (-8%) across the full weaning period (LD 0–21) were observed. These bodyweight changes in the F_2 generation were considered a treatment-related effect.

At 1800 ppm, the mean initial birth weight was statistically significantly decreased in the F_1 generation (-8%, both sexes) relative to controls. While all F_1 pups gained weight during the weaning period, the mean bodyweight gain was notably decreased compared with controls (-14%, M&F combined). In the F_2 generation, mean initial birth weight and pup weights at the LD4 cull were comparable to controls. However, from LD7 onwards, pup bodyweights were notably decreased (-8 to -14%; statistically significant at LD14 onwards), resulting in overall decreased bodyweight gains between LD 0–21 compared with controls (-14%, M&F combined). The body weight differences observed at 1800 ppm were considered treatment-related and adverse.

Table KIIA 5.6.1/02-16: Mean F_1/F_2 Pup Weights during weaning (g)

Lactation day	F1 (ppm)				F2 (ppm)			
	0	**100**	**500**	**1800**	**0**	**100**	**500**	**1800**
Male pups								
0	6.2±0.11	6.2±0.11	6±0.1	5.7±0.12 (-8%)**	6.2±0.1	6±0.11	6±0.11	6±0.08
4b	10.4±0.24	10.4±0.24	9.9±0.28	9.4±0.29* (-10%)	10.3±0.23	9.7±0.23	9.8±0.26	9.8±0.22
4c	10.4±0.25	10.4±0.24	9.9±0.28	9.4±0.29* (-10%)	10.3±0.23	9.7±0.22	9.8±0.26	9.8±0.21
7	16.2±0.3	16.3±0.34	15.5±0.41	14.4±0.39** (-11%)	16.3±0.33	15.8±0.29	15.5±0.38	15±0.4 (-8%)
14	32.5±0.42	32.1±0.62	30.8±0.7	28.2±0.53** (-13%)	32.4±0.64	31.5±0.48	30.3±0.51* (-6%)	28.4±0.76** (-12%)
21	49.8±0.59	49.3±0.96	48.0±1.0	43.5±0.78** (-13%)	49.6±0.8	48.1±0.086	45.9±0.74** (-7%)	43.8±1.19** (-12%)
BWG 0–21	43.6	**43.1**	**42.0**	**37.8 (-13%)**	43.4	**42.1**	**39.9 (-8%)**	**37.8 (-13%)**
Female pups								
0	5.9±0.12	5.9±0.12	5.7±0.11	5.4±0.11* (-8%)	5.9±0.11	5.8±0.09	5.7±0.1	5.6±0.08
4b	10±0.28	10.1±0.28	9.7±0.29	9.1±0.27 (-9%)	9.9±0.24	9.6±0.21	9.4±0.24	9.5±0.25
4c	10±0.29	10.1±0.28	9.7±0.29	9.1±0.26 (-9%)	9.9±0.24	9.6±0.21	9.4±0.24	9.5±0.25
7	15.8±0.36	15.8±0.39	15.1±0.41	14±0.35** (-11%)	15.7±0.34	15.4±0.22	15±0.35	14.4±0.39* (-8%)
14	31.9±0.52	31.3±0.6	30±0.71	27.7±0.51** (-13%)	31.8±0.57	30.9±0.4	29.6±0.52 (-7%)	27.5±0.76** (-14%)
21	48.4±0.72	47.8±0.8	46.1±1.05	42.2±0.7** (-13%)	47.8±0.77	46.8±0.71	44.4±0.74** (-7%)	41.8±1.07** (-13%)
BWG 0–21	42.5±	41.9	40.4	36.8 (-13%)	41.9	41.0	38.7 (-8%)	36.2 (-14%)
Combined (M&F) data								
BWG 0-4	4.2±0.18	4.3±0.16	3.9±0.20	3.7±0.18 (-12%)	4.1±0.15	3.8±0.14	3.8±0.17	3.8±0.17
BWG 4-7	5.8±0.12	5.8±0.15	5.5±0.17	5.0±0.13** (-14%)	5.9±0.14	5.9±0.12	5.6±0.16	5.0±0.22** (-15%)
BWG 7-14	16.2±0.23	15.8±0.33	15.2±0.40	13.8±0.25** (-15%)	16.1±0.39	15.6±0.26	14.7±0.26* (-9%)	13.2±0.41** (-18%)
BWG 4-21	39.0±0.48	38.6±0.70	37.5±0.82	33.5±0.51** (-14%)	38.7±0.64	37.7±0.61	35.6±0.58** (-8%)	33.0±0.96** (-15%)
BWG 14-21	17.0±0.27	17.0±0.34	16.7±0.39	14.8±0.29** (-13%)	16.7±0.31	16.2±0.41	15.3±0.34* (-8%)	14.7±0.41** (-12%)
BWG 0–21#	43.2	42.9	41.4 (-4%)	37.3** (-14%)	42.7	41.5	39.4** (-8%)	36.8** (-14%)

'4b' and '4c' denote bodyweights on day 4 before and after culling, respectively. */** - denotes statistically significant at the *p≤0.05 or **p≤0.01 level. # Combined sex data for bodyweight gain from GD 0 to GD 21 is derived from data presented on pages 105–108 of the study report (no standard errors were provided). Other data excerpted from pages 40–41 and 93–104 of the study report, except for per cent change from control and rounded to integers, which was undertaken by the evaluator using Microsoft Excel.

4. Gross pathology, organ weights, and histopathology findings

No test substance-related macroscopic or microscopic lesions were observed at necropsy in F_1 and F_2 litters, and organ weight changes were confined to decreased absolute weights for brain (-3 to -4%) at 1800 ppm (along with increased relative-to-bodyweight brain weights

of +10 to +12% at this dose), as well as decreased absolute thymus (-7 to -9%) and spleen (-5 to -15%) weights in pups at 500 and 1800 ppm (with no change in relative organ weights). These findings were considered secondary consequences to decreased bodyweights in pups at 500 and 1800 ppm, and not toxicologically adverse.

5. Sexual maturation

Anogenital distance was unaffected by the test substance in F_2 pups.

Treatment-related delays in the onset of vaginal patency (36.1 in controls vs 34.3 days; not statistically significant) and preputial separation (46.5 in controls vs 42.6 days; $p \leq 0.01$) were observed in 1800 ppm F_1 animals retained for second generation production (Table KIIA 5.6.1/02-17). The study author regarded these findings as secondary to lower bodyweights in these animals during lactation, with subsequent delays in sexual maturation. The evaluator considered these conclusions to be appropriate.

Table KIIA 5.6.1/02-17. F1 Sexual Maturation

Dose (ppm)	0	100	500	1800
	males			
Preputial separation days n % reaching BW deficit on Day 21 **days delayed**	 42.6±0.41 29 100 -	 42.5±0.52 27 100 -	 43.3±0.34 27 100 4%	 46.5±0.56** 27 100 13%** **3.9**
	females			
Vaginal opening days n % reaching BW deficit on Day 21 **days delayed**	 34.3±0.61 27 97	 34.0±0.57 26 100	 34.1±0.49 27 100	 36.1±0.72 28 100 13% **1.8**

III. CONCLUSIONS

Under the conditions of the study, BYI 02960 produced no effects on clinical signs, gross necropsy findings, reproductive organs, sperm parameters, or gestation length in both reproductive generations.

In parental observations, clear treatment-related decreases in mean bodyweight and bodyweight gain were observed at 1800 ppm in P_1 females. Apparent bodyweight effects in P_2 males and females during the pre-mating phase at 1800 ppm reflected the lower initial bodyweight stemming from the delayed post-natal growth observed in F_1 offspring at this dose. Decreased food consumption was noted at 1800 ppm females in both generations during the pre-mating period..

Decreased bodyweight gain was observed during the gestation period at 1800 ppm in both generations, but the percent bodyweight gains relative to the GD 0 body weight , which account for carry-over effects from the pre-mating period resulting in lower starting bodyweight during gestation, were unaffected by treatment. However, the lactation period in both generations reported increased bodyweight gains in 1800 ppm females, suggesting a potential marginal recovery in bodyweight gain compared with concurrent controls.

While reproductive parameters were unaffected by treatment in P_1 females, a slight decrease (-17%) in the number of estrus cycles and a slight decrease (-8%) in the total number of implantation sites

were noted in 1800 ppm P_2 females, which may be associated with the decreased bodyweights observed at the same dose. In addition, a slight reduction in epididymal sperm count in P_1 and P_2 males and in testicular sperm count in P_2 males was observed at 1800 ppm.

In terminal observations, treatment-related histopathological changes were noted in the liver (centrilobular hepatocellular hypertrophy) in P_1 males, and accompanied by slight (+9%) increases in the absolute and relative liver weights, though these changes were considered an adaptive effect in the context of this study. While statistically significant increases in absolute and relative liver weights were also noted in other 1800 ppm animals across both generations, no other treatment-related liver histopathological findings were observed. An increase in the relative thyroid weight in both P_1 and P_2 males was not accompanied by gross or histopathological change, and the toxicological significance is uncertain.

Ovarian follicle counts in F_1 females selected as P_2 maternal animals were unaffected by treatment with the test substance.

In fetal observations, general survival parameters were unaffected by treatment in both generations. A slight decrease (-15%) in overall litter size in F_2 pups at 1800 ppm was noted, and when considered along with decreased (-8%) overall implantations in P_2 dams, suggests a slight effect on reproductive function at 1800 ppm.

Pup body weights were unaffected by treatment at 100 ppm in both generations. Treatment-related and statistically significant decreases in mean bodyweight (-7 to -14%) and bodyweight gain (-8 to -14%) were observed during the weaning period in both generations at 1800 ppm, and in the F_2 generation at 500 ppm. In the F_2 generation at 500 ppm, the bodyweights were not affected until the latter part of lactation (starting lactation day 14). Terminal findings (necropsy, histopathology, and organ weight analyses) did not show treatment-related effects in both generations.

Treatment-related changes in sexual maturation were limited to slight delays in vaginal patency and preputial separation at 1800 ppm in F_1 pups; these effects were considered secondary to decreased mean bodyweights in these animals during weaning.

The **No Observed Adverse Effect Level (NOAEL) for parental toxicity was 500 ppm (38.7 mg/kg bw/d); the LOAEL was 1800 ppm (137 mg/kg/day) based** on decreased bodyweight (-10%) and bodyweight gain. (-43%) in P_1 females during the pre-mating period at 1800 ppm.

The **NOAEL for offspring toxicity was 100 ppm (7.7 mg/kg bw/day); LOAEL was 500 ppm (38.7)** based on decreased bodyweight (-7%) in the F_2 male and female pups.

The **NOAEL for reproductive toxicity was 500 ppm (38.7 mg/kg bw/day); LOAEL was 1800 ppm (137 mg/kg/day)** based on a 17% lower ($p \leq 0.05$) number of estrus cycles. In addition, a slight reduction in epididymal sperm count in P_1 and P_2 males and in testicular sperm count in P_2 males was observed at 1800 ppm.

This study was classified as reliable (acceptable/guideline) and satisfied the guideline requirements (OPPTS 870.3800; OECD N0. 416; MAFF 12 Nousan 8147) for a reproduction and fertility study.

Primary review: Australia
Secondary review: PMRA and US EPA

Report: Cada A (2012) A Chronic Toxicity Feeding Study in the Beagle Dog with Technical Grade BYI 02960. Xenometrics, LLC, Stilwell, Kansas. Study No**.:** 09-C76-RZ. 17 February 2012. MRID 48844121. PMRA 2239521. Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor report number: M-425272-01-2.

Guidelines: OPPTS 870.4100 (1998)
OECD 452 (1981)

Deviations: Pre-dosing coagulation analysis was not undertaken. All other deviations were of a minor nature. Overall, the deviations are considered not to have affected the reliability of the study.

GLP: Yes Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a 1-year dietary study (MRID 48844121), BYI 02960 (96.2%; batch 2009-000239) was administered to 5 to 6-month old beagle dogs (4/sex/dose) at concentrations of 0, 150, 300, or 1000 ppm (0/0, 4.6/4.1, 7.8/7.8, and 28.1/28.2 mg/kg bw/d for M/F, respectively) for one year. Parameters evaluated included bodyweight, bodyweight gain, food consumption, test compound intakes, clinical signs, haematology, coagulation, clinical chemistry, urinalysis, ophthalmology, organ weights, and gross and microscopic pathology. In addition, after an overnight fast blood samples were collected from 1/sex at 0 and 300 ppm animals on study day 141 and analysed for test substance concentrations in plasma.

Under the conditions of this study, there were no test substance-related effects on clinical signs, survival, haematology, coagulation, clinical chemistry, urinalysis, ophthalmology, general macroscopic findings and organ weight parameters.

Changes in bodyweight gain were confined to females at 1000 ppm, with an 86% lower bodyweight gain relative to controls in study week 1, and 17 to 66% lower relative gains over the first two 13-week intervals and the final 26 week interval of the study. This decreased bodyweight gain resulted in terminal bodyweights being 9% lower in females at this dose relative to controls. The decreased bodyweight gain in females at 1000 ppm during week 1 may partially be linked to a 26% lower food consumption relative to controls over the same period. The decease decrease in absolute body weight was slight and showed no statistical significance. Therefore, the changes in body weight were not considered to be adverse. In addition, group mean food consumption was unaffected when averaged across the whole one year treatment period.

Blood plasma levels of BYI 02960 in animals in the 300 ppm dosing group on study day 141 peaked at 3 hours after withdrawal of food. A slightly elevated blood plasma concentration of test substance was observed in males compared with females.

Test-substance related histopathological findings were confined to myofiber degeneration in skeletal muscle, particularly in gastrocnemius muscle at a 50% incidence and in bicep muscle at 75% incidence in both sexes receiving 1000 ppm diets. These lesions were of either minimal or slight severity (each at 1 or 2 out of a maximum severity of 5). All animals presenting with gastrocnemius muscle degeneration also presented with biceps femoris muscle lesions, giving a total incidence for

muscle histopathology of 75% in both sexes at 1000 ppm. Degeneration of the myofiber comprised of one or more of the following changes: atrophy, necrosis, and/or presence of inflammatory cells around the affected myofiber. In addition, there was a slight increase in ALT level.

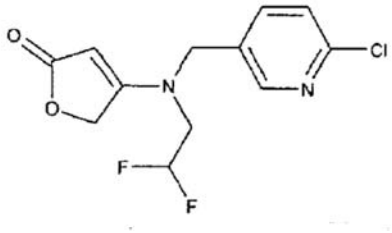
The no-observed-adverse-effect level (NOAEL) was 300 ppm (7.8 mg kg bw/d for both M/F) for both sexes. The LOAEL was 1000 ppm (28.1/28.2 mg kg bw/d for M/F) for both sexes, based on increased incidence of minimal-to-slight, focal to multifocal areas of skeletal muscle degeneration in gastrocnemius and biceps femoris (75% combined incidence) in both sexes with associated slight increase in ALT level.

This study is fully reliable (acceptable/guideline) and satisfies the data requirements for a chronic toxicity study in dogs (OPPTS 870.4100; OECD 452).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   4-[(6-chloropyridin-3-ylmethyl)(2,2-difluorethyl)amino]furan-2(5H)-one



Origin batch #:	2009-000239
Purity:	96.2% (^{1}H-NMR)
Description:	Beige powder
CAS #	Not reported
Stability of test compound:	Stored in the dark at 18 to 26°C. Stated to be stable for two years if stored at 25+5°C (date of analyses 16 January 2009 and 14 Jan 2011). Samples of feed were analysed for test material concentrations and found to be acceptable.

2. Vehicle: Corn oil and acetone prior to incorporation into diet.
3. Test animals

Species:	Dogs
Strain:	Beagle
Age at dosing:	5 to 6 months
Weight at dosing:	6.3 to 7.7 kg (males) & 6.2 to 8.1 kg (females)
Source:	Marshall BioResources, North Rose, New-York, USA
Acclimation period:	At least six days
Diet:	Purina Mills Lab Canine Diet Etts 5006-3 *ad libitum* except for fasting prior to blood collection.
Water:	Tap water, *ad libitum*
Housing:	Individually in stainless steel runs.

4. Environmental conditions

Temperature:	18 to 29°C
Humidity:	30 to 70%
Air changes:	At least 12.15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. In-life initiated/completed

   07-Jan-2010 to 28-Jan-2011

2. Animal assignment and treatment

   Details of animals and dosing levels are shown in Table KIIA 5.3.4/01-1 and KIIA 5.3.4/01-2. Four groups of 4 dogs/sex/dose received concentrations of 0, 150, 300, or 1000 ppm BYI 02960 in *ad libitum* portions of feed for at least one year, with fresh feed provided daily. After the one year dosing period, animals were fasted overnight, sacrificed, and subjected to necropsy.

   The dosing regimen was based on results from the 90-day dietary study in dogs (Eigenberg, 2010; Study No. 09-S76-QQ) where the study authors regarded the LOAEL to be 1200 ppm (33/41 mg kg bw/d for M/F), based on decreased bodyweight and myofiber atrophy/degeneration. The NOAEL was regarded to be 400 ppm (12 mg kg bw/d for both sexes).

   Achieved mean daily test substance intakes at 0, 150, 300, and 1000 ppm were 0/0, 4.6/4.1, 7.8/7.8, and 28.1/28.2 mg/kg bw/d for M/F, respectively. Data for mean test substance intakes over each study week and standard deviations within groups were provided.

   Animals were randomised by sex to dosing groups using computer software (Instem Datatox®) such that bodyweights in any one group deviated by less than 20% from the group mean.

   Animal husbandry complied with or exceeded requirements outlined in Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy Press, Washington, D.C. 1996).

Table KIIA 5.3.4/01-1: Study design

nominal conc. in diet (ppm)	achieved conc. in diet (ppm) [±SD]	achieved mean daily intake (mg/kg bw/d)[A] [±SD]		animal ID No's [N=4]	
		M	F	M	F
0	0	0	0	RZ0001 to 0004	RZ0101 to 0104
150	145±5	4.66±0.61	4.11±0.68	RZ1001 to 1004	RZ1101 to 1104
300	290±14	7.84±1.19	7.81±1.26	RZ2001 to 2004	RZ2101 to RZ2104
1000	975±33	28.21±3.95	28.18±3.18	RZ3001 to 3004	RZ3101 to RZ3104

[A]- Estimates of exposure were based on measured concentrations of test substance in feed and measured feed intakes. SD for mean intakes were calculated by the evaluator and exclude SD values for within-group weekly means (*i.e.*, is an underestimate). Data excerpted from pages 10, 25, 31, and 32 of the study report, except for standard deviations which were calculated by the evaluator.

Table KIIA 5.3.4/01-2: Group weekly mean BYI 02960 consumption.

Day	Dose (ppm)							
	M				F			
	0	**150**	**300**	**1000**	**0**	**150**	**300**	**1000**
7	0	6.52	11.37	34.14	0	5.39	11.35	30.14
14	0	5.57	10.36	33.81	0	5.16	10.50	31.92
21	0	6.22	10.19	35.40	0	5.82	10.71	33.40
28	0	5.62	10.36	34.57	0	5.56	9.79	33.61
35	0	5.62	9.79	35.97	0	5.53	9.78	31.80
42	0	5.31	9.69	34.47	0	5.07	9.23	31.68
49	0	5.26	9.77	33.37	0	5.15	9.05	31.86
56	0	5.27	9.78	33.20	0	5.05	9.45	32.76
63	0	5.17	9.11	31.65	0	4.64	8.90	32.64
70	0	5.41	8.93	32.98	0	4.95	8.81	30.26
77	0	4.96	8.64	31.58	0	4.74	9.17	28.39
84	0	5.28	8.30	32.44	0	4.76	8.71	31.69
91	0	4.92	7.75	30.32	0	4.63	8.33	28.75
98	0	4.75	7.67	29.70	0	4.09	8.13	26.61
105	0	4.84	7.80	30.92	0	4.05	8.06	28.38
112	0	5.12	8.20	32.96	0	4.05	8.98	33.25
119	0	5.02	8.10	31.56	0	4.30	8.91	30.60
126	0	4.47	7.52	29.60	0	4.05	8.00	24.03
133	0	4.75	7.42	31.34	0	4.39	6.91	22.94
140	0	4.93	7.83	31.21	0	4.20	7.55	26.39
147	0	4.39	7.70	28.26	0	3.34	7.74	28.29
154	0	5.07	8.06	28.98	0	4.02	7.95	29.17
161	0	4.66	7.52	26.59	0	3.86	7.72	29.20
168	0	4.69	7.61	27.78	0	4.15	7.76	29.94
175	0	4.40	6.69	26.62	0	4.00	6.21	27.36
182	NA	NA	NA	NA	0	4.05	6.65	24.76
189	0	4.09	7.31	25.01	0	3.40	5.96	23.39
196	0	4.11	7.47	27.49	0	3.65	6.91	27.12
203	0	4.34	7.43	26.04	0	3.68	6.94	29.40
210	0	4.01	7.03	25.90	0	3.86	7.63	31.45
217	0	4.17	7.11	26.45	0	3.79	7.50	29.73
224	0	3.77	6.76	25.90	0	3.92	8.00	30.35
231	0	4.38	7.35	26.71	0	4.22	7.76	31.26
238	0	4.28	6.85	24.90	0	4.26	7.60	28.38
245	0	4.10	6.91	24.19	0	3.93	7.47	30.37
252	0	4.02	7.01	24.73	0	3.70	7.66	29.46
259	0	4.25	7.19	24.37	0	3.72	6.85	25.51
266	0	4.40	7.65	25.73	0	3.93	7.65	27.86
273	0	4.12	7.23	25.04	0	3.91	7.58	26.26
280	0	3.81	6.71	22.26	0	3.57	6.82	24.26
287	0	4.12	6.94	25.96	0	3.65	6.67	24.47
294	0	4.22	6.97	25.03	0	3.83	6.89	28.77
301	0	4.28	6.69	23.67	0	3.48	6.07	25.90
308	0	4.52	7.09	23.79	0	3.72	6.67	21.87
315	0	4.10	6.47	23.63	0	3.37	7.19	25.62
322	0	4.15	6.82	23.41	0	3.50	7.39	24.61
329	0	4.19	6.72	23.90	0	3.19	6.75	22.09
336	0	4.61	6.90	23.75	0	3.29	6.65	25.75
343	0	4.23	7.34	24.86	0	3.04	5.87	27.10
350	0	4.35	6.95	25.00	0	3.41	6.08	26.75
357	0	4.25	6.90	23.59	0	3.13	6.24	24.10
364	NA	NA	NA	NA	0	3.38	7.02	23.91
Day 1 – 364	0	**4.66±0.61**	**7.84±1.19**	**28.21±3.95**	**0**	**4.11±0.68**	**7.81±1.26**	**28.18±3.18**

SD excludes SD values within group weekly means (*i.e.*, is an underestimate) Data excerpted from pages 326 and 327 of the study report, except for the D1– 364 mean, which was calculated by the evaluator using Microsoft Excel.

3. Diet preparation and analysis

To prepare admixed diet, BYI 02960 was dissolved with acetone and corn oil in an Erlenmeyer flask. The test substance mixture was then transferred to a separatory funnel and added to the diet while the diet was being mixed with a Hobart mixer. After the test substance mixture was added to the diet, the Erlenmeyer flask and separatory funnel were rinsed with acetone and the rinse added to the ration. Acetone carrier was assumed to evaporate during the 10 min mixing time. Control diets were admixed with vehicle only.

Homogeneity and stability analyses of test substance in diet were previously described in Moore (2008) as part of the 90-day dietary study with BYI 02960 in the beagle dog (Eigenberg, 2010). Briefly, the stability and homogeneity of BYI 02960 in the diet after freezer storage was determined on weeks 1, 2, 3, 6, 11, and 14. The test material in the diet was found to be homogenous (96–100% of nominal levels; relative standard deviations for sample from the top, middle and bottom layers of 3.8-3.9%) and stable for 7 days at room temperature (100–104% of nominal levels) as well as being stable for 35 days at freezer temperature (95–102% of nominal levels).

In the current study, the concentration of test substance in feed was verified using a validated HPLC analysis method weekly for the first three weeks of the study, and monthly thereafter. Overall, prepared diet was 97–98% of nominal concentrations during the course of the study.

Further details of feed batch preparation intervals and storage were not provided. However, the evaluator notes that the same facility conducted both the 90-day and one-year toxicity studies in beagle dogs, and that the stability and homogeneity of the prepared diet used during this present study has been sufficiently demonstrated. As a result, the omission of additional storage and feed batch preparation details is not expected to affect study validity in this case.

4. 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 histopathologic lesion evaluations that were one-tailed.

Continuous data were analyzed by Bartlett's Test for homogeneity. These data were found to be homogeneous and subsequently had an Analysis of Variance (ANOVA) performed followed by Student's *t*-Test on parameters showing a significant effect by ANOVA. Frequency data (*i.e.*, micropathology incidence, etc.) that were examined statistically were evaluated using the Chi-Square and/or Fisher Exact Tests.

C. METHODS

1. General Observations

Observations for clinical signs of toxicity were undertaken twice daily (mornings and afternoons/evenings) on weekdays and once daily on weekends and public holidays. Detailed physical observations were undertaken on the day of first initiation dosing and on a weekly basis thereafter.

2. Body weights

All animals were weighed at study initiation and at necropsy, and on a weekly basis over the study period.

3. Food consumption, food efficiency, and daily intake of test substance

Food consumption was recorded daily during the study. Food use efficiency data were not presented in this study. Final mean weekly intake of test substance is detailed in Table KIIA 5.3.4/01-1 above.

4. Clinical pathology (hematology, urinalysis and clinical chemistry)

Hematology, clinical chemistry, and urinalysis evaluations were performed on all animals once immediately prior to Study Day 1 and at approximately 3, 6, 9, and 12 months during the dosing period. Coagulation parameters were not collected pre-treatment, but this omission was not considered to affect study validity.

Hematology and coagulation

Animals were fasted overnight and blood (approx. 8 mL/animal) was collected by puncturing the jugular vein and storing the samples in Vacuette® or similar tubes containing potassium EDTA for hematology, serum separator tubes for clinical chemistry, and sodium citrate for coagulation parameter analysis.

The following haematology and clinical chemistry parameters were measured:

Hematology

Red blood cell count	Hemoglobin
Reticulocyte count	Hematocrit
Platelet count	Mean corpuscular (cell) volume
White blood cell count	
Differential white blood cell count	Prothrombin time
Activated partial thromboplastin time	Mean corpuscular hemoglobin
Red blood cell distribution width	
Haemoglobin distribution width	Red blood cell morphology
Mean corpuscular (cell) hemoglobin concentration	

Clinical chemistry parameters

aspartate aminotransferase	total protein
alanine aminotransferase	albumin
alkaline phosphatase	total bilirubin
creatinine phosphokinase	lactic dehydrogenase
urea nitrogen	creatinine
total cholesterol	triglycerides
glucose	chloride
sodium	potassium
calcium	inorganic phosphorous
gamma-glutamyltransferase	gobulin
uric acid	A/G ratio

Urinalysis parameters

Urine collected from a metabolism pan under each animal's run was collected over a 24 hour period, and analysed for the following parameters:

appearance	ketone
24 hour urine volume	protein
specific gravity	bilirubin
pH	blood
sediment	urobilinogen
glucose	leukocytes
nitrite	

5. Concentrations of test substance (but not metabolites) in plasma

All animals were fasted overnight on study day 140. On study day 141, food was re-supplied to animals but then withdrawn after one hour in four animals (1/sex in control and 300 ppm groups). Blood was collected at 1 and 8 hours after food removal for the control animals, and at 1, 3 and 8 hours after food removal for the 300 ppm animals. Blood sampled from the jugular vein was collected into tubes containing lithium heparin, stored on ice, and then centrifuged at 3000 rpm at 2–4°C within 30 minutes of collection to obtain plasma. Plasma was then stored at -70°C until analysis of test substance concentrations by HPLC.

6. Ophthalmology

Ophthalmological analyses of each eye of all animals were performed during acclimation and immediately prior to sacrifice on study days 365 to 367. A mydriatic agent (1% Mydriacyl) was used to dilate pupils to assist examinations. The cornea, aqueous humor, and lens were examined using a KOWA SL-15 slit lamp microscope. The vitreous humor, retina, choroid, and optic disc were examined with a Heine Omega indirect ophthalmoscope (Heine USA Ltd, NH) and a condensing lens. Examinations were performed in a semi-darkened room.

7. Sacrifice and pathology

On study days 365 to 367, all animals were sacrificed by intravenous injection of Fatal-Plus® and subjected to gross necropsy. All tissues from control and high-dose (1000 ppm) groups were processed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under a light microscope (**Table KIIA 5.3.4/01-3)**. Tissues collected from all animals were stored for potential examination in the event of histopathological findings being found in the high dose group.

Table KIIA 5.3.4/01-3: Organs/tissues collected for pathological examination

Tissue [N=4/sex for controls and high dose]	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted[a]
Adrenal gland	X	X	X
Aorta		X	X
Bone marrow		X	X
Bone (sternum)		X	X
Bone (rib/cc jct)		X	X
Brain (cerebrum, midbrain, cerebellum, medulla/pons)	X	X	X
Cervix		X	X
Epididymides	X	X	X
Eosophagus		X	X
Eye (with optic nerve)		X	X
Heart	X	X	X
Kidney	X	X	X
Intestines (duodenum, jejunum, ileum, cecum, colon, rectum)		X	X
Larynx/pharynx		X	X
Liver with gallbladder	X	X	X
Lung	X	X	X
Lymph nodes (retropharyngeal, mesenteric)		X	X
Mammary gland		X	X
Nasopharynx		X	X
Ovary	X	X	X
Oviduct		X	X
Pancreas		X	X
Parathyroid		X	X
Pituitary	X	X	X
Prostate	X	X	X
Salivary glands		X	X
Sciatic nerve		X	X
Skeletal muscle (gastrocnemius, biceps femoris)		X	X
Skin		X	X
Spinal cord, (cervical, lumbar, thoracic)		X	X
Spleen	X	X	X
Stomach		X	X
Testis	X	X	X
Thymus	X	X	X
Thyroid with parathyroid	X	X	X
Trachea		X	X
Ureter		X	X
Urinary bladder		X	X
Uterus	X	X	X
Vagina		X	X
Gross lesions		X	X

Data excerpted from page 23 of the study report.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

   There was no test substance related effect on clinical signs.

2. Mortality

   There were no unscheduled mortalities.

B. BODY WEIGHT AND BODY WEIGHT GAIN

All groups gained mean bodyweight throughout 1-year study period. Changes in bodyweight gain were confined to females at 1000 ppm, where decreased bodyweight gain relative to controls (86% lower) was seen in study week 1, and gains remained 17 to 66% lower over the first two 13-week intervals and the final 26 week interval of the study. This resulted in terminal bodyweights being 9% lower in females at this dose relative to controls. However, US EPA reviewer believed the decease was slight and showed no statistical significance. Therefore, the body weight changes were not considered to be adverse. There was no effect on terminal bodyweights in any other group

Table KIIA 5.3.4/01-4). **Table KIIA 5.3.4/01-4: Bodyweights and bodyweight gain (kg) in beagle dogs during treatment**

	Males				Females			
Dose (ppm)	**0**	**150**	**300**	**1000**	**0**	**150**	**300**	**1000**
Initial BW (Day_0)	7.2±0.5	7.0±0.5 **(-4%)**	7.1±0.5 **(-1%)**	7.0±0.4 **(-2%)**	7.1±0.7	7.3±0.7 **(+3%)**	7.3±0.5 **(+3%)**	7.3±0.4 **(+3%)**
BWG Week 1 (Days 0–7)	0.2±0.1	0.3±0.3	0.2±0.1	0.2±0.1	0.3±0.2	0.2±0.1	0.3±0.4	0.04±0.2 ***(-86%)***
BWG Weeks 1–13	2.3±1.1	2.2±1.3	2.7±0.5	2.8±0.9	1.7±0.3	1.9±0.5	2.0±0.5	1.4±0.4 ***(-17%)***
BWG Weeks 13–26	1.1±0.3	1.3±0.2	1.0±0.3	1.6±0.4	0.9±0.2	0.7±0.4	0.7±0.4	0.3±0.4 ***(-66%)***
BWG Weeks 26–52	0.5±0.6	0.2±0.3	0.6±0.5	0.8±0.5	0.5±0.4	0.4±0.1	1.0±0.6	0.2±0.2 ***(-60%)***
BWG Weeks 1–52	3.9±1.8	3.6±1.6	4.2±1.2	5.3±1.6	3.0±0.6	3.0±0.5	3.7±1.0	2.0±0.9 ***(-33%)***
Final BW (Day 364)	11.1±2.1	10.5±2.1 **(-5%)**	11.3±1.4 **(+2%)**	12.3±1.9 **(+10%)**	10.2±0.7	10.3±1.1 **(+1%)**	11.0±0.7 **(+8%)**	9.2±0.7 **(-9%)**

Data excerpted from page 26 of the study report, except for per cent change from control for bodyweight gain in females at 1000 ppm, which was calculated by the evaluator using Microsoft Excel.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

The decreased bodyweight gain in females at 1000 ppm during week 1 may partially be related to a 26% lower food consumption (not statistically significant) relative to controls during the same period (Table KIIA 5.3.4/01-5). However, group mean food consumption was unaffected in all other groups and unaffected in females at 1000 ppm during weeks 2 to 52, and when calculated as an overall mean during the one year treatment period. Food use efficiency was not calculated by the study authors.

Table KIIA 5.3.4/01-5: Mean Food consumption (g/day) in beagle dogs

Recording interval	Dose (ppm)							
	M				F			
	0	150	300	1000	0	150	300	1000
Days 0–6	282	319 **+13%**	284 **+1%**	249 **-12%**	304	274 **-10%**	289 **-5%**	225 **-26%**
Weeks 1–52	289	313 **+8%**	275 **-5%**	308 **+7%**	253	267 **+6%**	262 **+4%**	257 **+2%**

Data excerpted from page 26 of the study report.

D. CLINICAL PATHOLOGY

1. Haematology and coagulation

Haematology and coagulation findings were unremarkable.

2. Clinical chemistry

A slight, statistically significant increase in mean alanine aminotransferase (ALT) (23%; $p<0.05$) was observed at study termination in males from the 1000 ppm dose group when compared to controls. This slight increase may be biologically significant because the histopathology data showed that there was an increase in the incidence of myofiber degeneration in the skeletal muscle. Alanine aminotransferase was present in the skeletal muscle.

3. Urinalysis

Urinalyses parameters were unaffected by dosing.

E. TEST SUBSTANCE CONCENTRATIONS IN PLASMA

On study day 141, blood plasma levels of BYI 02960 peaked 3 hours after withdrawal of diet, with a slightly elevated blood plasma concentration of test substance in males compared with females (Table KIIA 5.3.4/01-6). The maximum concentrations in individuals were 3.00/2.26 µg/mL (M/F). There was no test substance detected in control group animals.

Table KIIA 5.3.4/01-6: Test substance concentrations (µg/mL) on study day 141

	Dose (ppm)									
	0	0	0	0	300	300	300	300	300	300
Animal No	RZ0001	RZ0101	RZ0001	RZ0101	RZ2001	RZ2101	RZ2001	RZ2101	RZ2001	RZ2101
Sex	M	F	M	F	M	F	M	F	M	F
Food (g) consumed within one hour of sampling	18	70	n/a	n/a	75	94	n/a	n/a	n/a	n/a
Time (h)	1	1	8	8	1	1	3	3	8	8
BYI 02960 conc. (µg/mL)	ND	ND	ND	ND	**2.57**	**1.7**	**3**	**2.26**	**1.63**	**1.11**
% difference M/F	ND	ND	ND	ND	-	**-34**	-	**-25**	-	**-32**

'ND' – denotes not detected. 'n/a' – denotes not available. Per cent difference between sexes were calculated by the evaluator using Microsoft Excel. Data excerpted from page 27 of the study report.

F. OPHTHALMOLOGY

Ophthalmology findings were unremarkable.

G. SACRIFICE AND PATHOLOGY

1. Organ weight

There was a trend toward increased absolute liver weights in males with increasing dose, with a 10% increase in males at 1000 ppm when compared to controls (not statistically significant). However, terminal body weights in high dose males were 8% higher than controls no corresponding trend in relative (to body weight) liver weight was noted in males, and no dose-related trend in liver weights was observed in females. Therefore, this change is not considered to be related to treatment. In females from the 1000 ppm dose group, there were 42% and 38% decreases in absolute and relative thymus weights when compared to controls (not statistically significant). The thymus weights were markedly low in 3 of 4 high dose females when compared to other test animals (absolute values of 3.8-5.8 g versus 8.2-16.8 g in control animals; relative values of 0.044-0.062% versus 0.092-0.174% in control animals). However, no corresponding pathology was recorded, and thymus weights in non-rodents are known to be quite variable; therefore, the decrease in thymus weights are not considered to be toxicologically relevant.

2. Gross pathology and histopathology

Gross necropsy findings were unremarkable.

Test-substance related histopathological findings were confined to myofiber degeneration in skeletal muscle at 1000 ppm, specifically in gastrocnemius at a 50% incidence (both sexes) and in biceps femoris muscle at 75% incidence (both sexes). These lesions were of either minimal or slight severity (each at 1 or 2 out of a maximum severity scale of 5). The animals with myofiber degeneration in gastrocnemius skeletal muscle also presented with biceps femoris muscle lesions, giving a total incidence of myofiber degeneration in gastrocnemius and/or biceps femoris muscle of 75% for both sexes at 1000 ppm.

Degeneration of the myofiber comprised of one or more of the following changes: atrophy, necrosis, and/or presence of inflammatory cells around the affected myofiber.

Table KIIA 5.3.4/01-7: Histopathology findings in beagle dogs at study termination

N=4	Males				Females			
	Dose (ppm)							
	0	**150**	**300**	**1000**	**0**	**150**	**300**	**1000**
Myofiber degeneration in gastrocnemius, minimal or slight severity [$\bar{x}$ score]	0	0	0	2/4 **(50%) [1.5]**	0	0	0	2/4 **(50%) [1.0]**
Myofiber degeneration in biceps femoris muscle, minimal or slight severity [$\bar{x}$ score]	0	0	0	3/4 **(75%) [1.3]**	0	0	0	3/4 **(75%) [1.0]**
Total incidence of myofiber degeneration (gastrocnemius and/or biceps femoris muscle)	-0	0	0	3/4 **(75%)**	0	0	0	3/4 **(75%)**

Statistical analyses not undertaken. Bracketed score denotes average severity of dogs with lesion, at a scale of 1 (minimal) to 5 (severe).
Data excerpted from page 28 of the study report, with the exception of total incidence which was calculated by the primary reviewer.

Based on decreased bodyweight gains (-33% vs controls across the treatment period) in females and minimal-to-slight, focal to multifocal areas of skeletal muscle degeneration in gastrocnemius and biceps femoris of both sexes, the study authors regarded the LOAEL in the study to be 1000 ppm (28.1/28.2 mg kg bw/d for M/F), with a NOAEL of 300 ppm (7.8 mg kg bw/d for both sexes) based

on the absence of adverse compound-related effects at 300 ppm. The primary reviewer agrees with this conclusion, also noting the 9% decrease in final bodyweight at study termination in 1000 ppm females compared with controls.

III. CONCLUSION

Under the conditions of this study, BYI02960 did not product effects on clinical signs, survival, haematology, coagulation, clinical chemistry, urinalysis, ophthalmology, or organ weight parameters.

The no-observed-adverse-effect level (NOAEL) was 300 ppm (7.8 mg kg bw/d for both M/F) for both sexes. The LOAEL was 1000 ppm (28.1/28.2 mg kg bw/d for M/F) for both sexes, based on, increased incidence of minimal-to-slight, focal to multifocal areas of skeletal muscle degeneration in gastrocnemius and biceps femoris (combined lesion incidence of 75% per sex) with associated slight increase in ALT level.

This study is fully reliable (acceptable/guideline) and satisfies the data requirements for a chronic toxicity study in dogs (OPPTS 870.4100; OECD 452)

Primary review: Australia
Secondary review: PMRA and US EPA

Report: Kennel P (2012) BYI 0260. Carcinogenicity study in the C57BL/6J mouse by dietary administration. Bayer SAS. Bayer CropScience. 355 rue Dostoïevski. BP 153, 06903 Sophia Antipolic Cedex, France. Laboratory Report No.: SA 08338. 24 February 2012. MRID 48844122. PMRA 2239506. Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N°TXRVP018. Bayer CropScience File No. M-425975-01-1.

Guidelines: OPPTS 870.4200 (1998)
OECD 451 (1981)
Commission Directive 88/302/EC Part B.32 (1987)
MAFF 12 Nousan 8147 (2000)

Deviations: Due to an oversight, observations for clinical signs were not undertaken on the last day of necropsy in the carcinogenicity group animals. Ten animals from various dosing groups and sexes potentially had no food available for two days during study week 50 (19 to 21 March, 2010).

GLP: Yes Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a carcinogenicity study (MRID 48844122), C57BL/6J mice received BYI 02960 (96.2%) in the diet at concentrations of 0, 70, 300, or 1500 ppm for 52 weeks (10/sex/concentration) or 78 weeks (50/sex/concentration). Achieved group mean daily intakes over the entire study duration were 0/0, 10.0/12.2, 43/53, and 224/263 mg/kg bw/d for M/F, respectively. Parameters evaluated included clinical signs, bodyweight, bodyweight gain, food consumption, test compound intakes, haematology, ophthalmology, organ weights, gross pathology (all groups) and histopathology (78-week group only). In addition, blood samples were collected and analysed for levels of parent compound at weeks 52 and 78.

There were no treatment-related effects on survival or clinical signs of toxicity across all dose levels tested. No adverse effects were observed at 70 and 300 ppm.

At 1500 ppm, treatment-related decreased mean body weights (-6%/-7% for M/F) and bodyweight gain (-19%/-13% for M/F) were observed compared with controls at statistically significantly levels. While food consumption in 1500 ppm animals was similar to controls, with only marginal decreases observed in females (-2–3% vs controls), the decreased mean bodyweight and bodyweight gain suggest decreased food use efficiency, and in this context, the changes in bodyweight and bodyweight gain are considered toxicologically adverse. However, the US EPA secondary reviewer considered the changes in the body weight parameters not approaching a magnitude of being adverse. In terminal findings at 1500 ppm, increased absolute and relative liver weights in males were observed and correlated to a toxicologically adverse change in liver histopathology (increased frequency and severity of diffuse hepatocellular vacuolation). Increased incidence of small/atrophic kidneys and decreased kidney weights were seen in 1500 ppm males.

Based on the study findings, the NOAEL was 300 ppm (43/53 mg/kg bw/d for males/females respectively). The LOAEL was 1500 ppm (224/263 mg/kg bw/d for males/females respectively), based on statistically significant increases in absolute and relative liver weights, with correlative

liver histopathology (diffuse hepatocellular vacuolation) and decreased kidney weights and alterations in kidney pathology in males (small/atrophic kidneys).

Under the conditions of this study, no treatment-related increases in tumor incidence were observed at all dose levels tested.

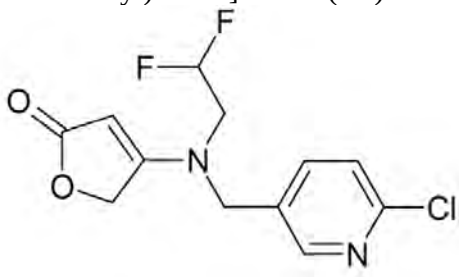
Blood plasma analyses indicated that the concentration of test substance in plasma scaled in an approximately linear manner with increasing dietary dose at both 52 and 78 week time points. There were minor sex differences in test substance concentrations at both time points, with males presenting with slightly elevated concentrations compared with females. However, no accumulation of test substance was noted during the study.

This study is classified as fully reliable (**acceptable/guideline**) and satisfies guideline requirements for a carcinogenicity toxicity in mice [OPPTS 870.4200; OECD 451 (1981)].

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one



Lot/Batch #:	2009-000239
Purity:	96.2% (^{1}H-NMR)
Description:	Beige powder
CAS #	951659-40-8
Stability of test compound:	Stated to be stable for two years if stored as recommended at 25±5ºC in an air tight, light resistant container. Further details regarding stability and homogeneity of test compound in admixed diet are described in Section I.B.3 below.

2. Vehicle and/or positive control: Acetone vehicle used to dissolve test compound prior to incorporation into diet. No positive control in this study.

3. Test animals

Species:	Mouse
Strain:	C57BL/6J
Age at dosing:	6 weeks old
Weight at dosing:	17.9 to 23.2 g for males; 13.9 to 19.6 g for females
Source:	Charles River Laboratories, St. Germain-sur-l'Arbresle, France
Acclimation period:	14 days
Diet:	A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*, except at designated time periods including an overnight fast prior to sampling periods and scheduled sacrifice. During the test period, test substance was incorporated into the feed of all animals except negative controls.
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Three per sex and per cage from arrival to pre-study day 8; and individually thereafter in suspended stainless steel and wire mesh cages.

4. Environmental conditions

Temperature:	20 to 24°C
Humidity:	40 to 70%
Air changes:	10 to 15/hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion

07 April 2009 to 20 Oct 2011 (main study). Provision of test substance diet started on 22 April, 2009.

2. Animal assignment and treatment

C57BL/6J mice (60/sex/concentration) received BYI 02960 in the diet at concentrations of 0, 70, 300, or 1500 ppm daily for 52 weeks (interim sacrifice: 10/sex/concentration) or 78 weeks (carcinogenicity phase; 50/sex/concentration; Table KIIA 5.5.3/01-1). Animals were kept on respective dietary concentrations of test substance while awaiting sacrifice (up to study week 80 for carcinogenicity phase animals). Achieved group mean daily intakes over the entire study duration were 0/0, 10.0/12.2, 43/53, and 224/263 mg/kg bw/d for M/F, respectively (Table KIIA 5.5.3-01/2). Dose levels and route of exposure were selected based on the results of the 90-day dietary toxicity study (Odin-Feurtet, 2012; Study No. SA 07295). In the 90-day study, a NOAEL was regarded as being established at 500 ppm for both sexes, with only marginally reduced (-3% maximum at study week 10) bodyweight gain in both sexes at this dose. A LOAEL of 2500 ppm was established for the 90-day dietary study, based on decreased body weight gains and potential food use efficiency decreases in males, toxicologically adverse liver-related clinical chemistry parameters, organ weight changes and histopathological changes in the liver in both sexes, and toxicologically adverse kidney-related clinical chemistry parameters, organ weight changes and histopathological changes in males at 2500 ppm.

In the current study, animals were examined three times during the acclimatisation period. At randomisation, animals were assigned to dose groups by computerised, stratified randomisation so that animals of each sex within the middle range of weight distribution were selected and that weights within each group were within ±20% of the mean on the day of randomisation. A negative control group received untreated diet. Animals not meeting weight criteria or physical condition requirements were removed from the study at randomisation, kept for one week in case of premature deaths in test group animals, then discarded as surplus to requirements.

Table KIIA 5.5.3/01-1: Study Design and Compound Intakes

Conc. in diet (ppm) [N=60]	Mean daily compound intakes (mg/kg bw/d) (A)		Animal No's 52 week interim sacrifice group [N=10]		Animal No's 78 week sacrifice group [N=50]	
	M	F	M	F	M	F
0	0	0	T1M2019 to 2028	T1F2079 to 2088	T1M2029 to 2078	T1F2089 to 2138
70	10.0	12.2	T2M2139 to 2148	T2F2199 to 2208	T2M2149 to 2198	T2F2209 to 2258
300	43	53	T3M2259 to 2268	T3F2319 to 2328	T3M2269 to 2318	T3F2329 to 2378
1500	224	263	T4M2379 to 2388	T4F2439 to 2448	T4M2389 to 2438	T4F2449 to 2498

Scheduled sacrifice was study week 52 (10/sex/dose interim sacrifice cohort) or study week 78 (remaining 50/sex/dose for carcinogenicity cohort). (A) - measured on a weekly basis for each animal in each study group. Data excerpted from page 22 of the study report. Standard deviations for test substance intakes were not provided by the study authors and were not calculated by the evaluator.

Table KIIA 5.5.3/01-2: Mean achieved dietary intake of BYI 02960 (mg/kg bw/d) over designated intervals

	Dose (ppm)					
	M			F		
	70	300	1500	70	300	1500
Wks 1-13	11.9	51	259	14.9	63	311
Wks 1-52	10.5	45	232	12.9	56	276
Wks 1-80	**10.0**	**43**	**224**	**12.2**	**53**	**263**

Data excerpted from page 36 of the study report.

3. Diet preparation and analysis

The test substance was added to small amounts of acetone to facilitate incorporation into the diet, with the acetone subsequently evaporating off during dry mixing into feed. Control diet was mixed with acetone vehicle only. Feed batches were 50 to 65 kg each, stored at ambient temperature when not in use, and prepared for use over at least an eight week period. In total, ten batches (F1 to F10) were prepared per dosing concentration. Homogeneity/concentration analysis by HPLC-UV at 260 nm was undertaken on 10 g samples of the first batch of each dosing concentration, and on the seventh batch of the 70 and 1500 ppm concentrations. For homogeneity analysis, three samples were taken from a different layer of the prepared batch (surface, middle, bottom). Achieved concentration analysis was also undertaken on a 10 g sample from the surface layer of the first, third, seventh, and ninth batches for each dosing concentration. Duplicate samples were obtained for concentration and homogeneity tests and stored at -20°C for potential future analyses. Surface level samples were also obtained from all control diet batches, with samples from the first, third, seventh, and ninth batches analysed for contamination by the test substance.

The study authors cited the stability of BYI 02960 in feed at ambient (room) temperatures for up to 110 days was verified at 2500 ppm in the chronic dietary (Garcin, 2012; Study No. SA 08337) and 28 day dietary studies in rats (Garcin, 2012; Study No. SA 09014). Additionally, stability analysis was conducted in the current study, with the fifth batch at 70 ppm analysed for concentrations after 71 and 110 days at room temperature.

Homogeneity analysis results indicated that test substance was found in diet at 87–96% of nominal concentrations at 70 ppm, 91–96% of nominal concentrations at 300 ppm, and 87–98% of nominal concentrations at 1500 ppm.

Concentration analysis results indicated that admixed test substance was present at 91–93% of nominal concentration at 70 ppm, 89–97% of nominal concentration at 300 ppm and 89–95% of nominal concentration at 1500 ppm.

Stability analysis of the 70 ppm test diet noted a slight degradation over time, with storage at room temperature resulting in a 4.2% decrease in nominal concentration after 110 days. Minimal degradation (0.2%) was noted in 2500 ppm test diet after 110 days at room temperature.

Mean extraction efficiency of spiked samples was 98.2% at 70 ppm, 95% at 300 ppm, and 94.5% at 1500 ppm spiked concentrations of BYI 02960 in feed, demonstrating that the analytical method used was appropriate.

Overall, based on the reported homogeneity, stability and concentration analyses, the evaluator considers that no adjustment factors for dietary concentrations are required in this case.

4. Statistics

Statistical methods employed in this study are listed in Table KIIA 5.5.3/01-3, and were carried out using SAS (Version 9.2), LTA.EXE or Pristima System (v 6.1.0 build 19) software. Survival analyses and statistical analyses of non-neoplastic and neoplastic lesions were performed on carcinogenicity phase animals only. Means and standard deviations were calculated for each group. All statistical analyses were carried out separately for males and females, and group means were compared at the 5% and 1% levels of significance.

Table KIIA 5.5.3/01-3. Statistics used in data analyses

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant (p>0.05)	**If preliminary test is significant (p≤0.05)**
Terminal bodyweight Bodyweight gain Haemotology (except rbc, platelets, wbc, neutrophils) Organ weight (absolute & relative[A])	**Bartlett Test**	Analysis of variance followed by 2-sided **Dunnett's test if significant**	**Kruskal-Wallis test** followed by 2-sided **Dunn's test if significant**
Bodyweight and mean food consumption per day parameters	**Bartlett Test**	Analysis of variance followed by 2-sided **Dunnett's test if significant**	**Bartlett Test** on log transformed data**;** if not significant then **ANOVA** on log transformed data, with 2-sided **Dunnett's test** on log transformed data if significant. If **Bartlett Test** on transformed data was significant then **Kruskal-Wallis test** followed by 2-sided **Dunn's test if significant**
Other haemotology (rbc, platelets, wbc, neutrophils)	**Bartlett Test**	One-way analysis of variance followed by 2-sided **Dunnett's test**	**Bartlett Test** on Square root transformed data**;** if not significant then **ANOVA** on transformed data followed by 2-sided **Dunnett's test** on transformed data if significant. If **Bartlett Test** on transformed data was significant then **Kruskal-Wallis test** followed by 2-sided **Dunn's test if significant**
Survival analysis	Adjusted mortality rates estimated using Kaplan-Meier estimation calculated separately for each sex and dose group. Statistical significance and dose related trends in survival were assessed using Cox's and Tarone's tests. Probabilities presented as 2-sided for pairwise comparisons and trend test.		
Clinical signs and macroscopic findings	Fisher's exact test (1-sided)		
Neoplastic and non-neoplastic findings	1-sided Cochran-Armitage method for trend and 1-sided Fisher's exact test for control vs treatment group comparisons. Cochran-Armitage trend significance was reported when any monotone response existed in the incidence data		

[A] - Relative organ weights (per cent of final body weight and per cent of brain weight) were calculated. Final body weights determined just prior to necropsy were used in the assessment of organ weight changes.

C. METHODS

1. Observations

Animals were observed at least once daily for clinical signs and twice daily for mortality/moribundity (once daily on weekends or public holidays). Detailed physical examinations (including palpation for masses) were conducted at least weekly. Data on clinical signs and masses were recorded. Due to an oversight, observations for clinical signs were not undertaken on the last day of necropsy in the carcinogenicity group animals: this omission was not considered to affect study integrity.

2. Body weights

All animals were weighed at least weekly during acclimatisation, then on study day 1, weekly for the first 13 weeks of the study, and every four weeks thereafter, with a final measurement prior to scheduled sacrifice.

3. Food consumption, food efficiency and daily intake

Food consumption was recorded weekly during the dosing period for the first 13 weeks of the study, then approximately monthly thereafter. Food use efficiency estimates were not provided.

4. Plasma concentration

During study weeks 52 and 78 at around 9 am, approx. 0.5 mL of blood from non-fasted animals (5/sex/dose for treated animals, 2 males from controls) was drawn from the retro-orbital venous plexus under isoflurane anaesthesia, collected into heparinised tubes and immediately frozen at -20^{o}C. Test substance concentrations in plasma were then determined by high performance liquid chromatography/tandem mass spectroscopy (HPLC-MS/MS) with an LOQ in plasma of 0.025 mg/L BYI 02960 (method 01331; MR-11/098). An internal standard for BYI 02960 (Batch#NLL 7780-47-4; Purity: 99.4%, various analysis methods on 26 Oct 2010. Expiry Oct' 2013) was used to correlate test substance levels. Examination of recovery rates of a mouse plasma sample series loaded with known BYI 02960 produced an overall mean recovery rate of 95±6.6%, indicating sufficient sensitivity for the analytical method used.

5. Clinical pathology (haematology only)

On study week 52, haematology analysis was undertaken on all surviving interim sacrifice animals and the first ten suitable animals/sex/dose from main study animals. Haematology analysis was also undertaken on the first twenty suitable animals/sex/dose from main study animals prior to necropsy at week 79 or 80. Animals were fasted overnight prior to blood sampling under isoflurane anaesthesia, with blood collected from the retro-orbital venous plexus and stored in tubes containing EDTA (0.5 ml). A blood smear was prepared from all animals not sampled for haematology at study termination, and from all animals at the respective sampling time points, and examined where unusual haematology results were reported. The following haematology parameters were examined:

Haematocrit	Mean corpuscular Hb
Haemoglobin (Hb)	Mean corpuscular Hb concentration
Erythrocyte count	Mean corpuscular volume (MCV)
Leukocyte count	Leukocyte differential count
Platelet count	

6. Sacrifice and pathology

Following overnight fasting and scheduled sacrifice by exsanguination under isoflurane anaesthesia on study weeks 53 (interim sacrifice group, study days 370, 371, or 372) or weeks 79/80 (study days 552 to 566), all animals were subjected to necropsy. Equivalent numbers of animals per group were sacrificed across each of the scheduled days. Additionally, any animals not surviving through to scheduled termination throughout the study period were subjected to necropsy. All significant macroscopic abnormalities were recorded, sampled and examined microscopically.

Necropsy included examination of external surfaces, all orifices, all major organs, tissues and body cavities (**Table KIIA 5.5.3/01-4)**. Macroscopic abnormalities were recorded and sampled, and together with tissue samples marked below from all main group animals (study week 78 scheduled sacrifice) were embedded in paraffin wax and subjected to histological analysis using haematoxylin and eosin staining. An exception was that exorbital lachrymal gland, larynx/pharynx and nasal cavities were not embedded in paraffin prior to analysis. Samples were fixed by immersion in 10% neutral buffered formalin with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative.

Initial examinations of macroscopic and microscopic lesions by the study pathologist (M Fouque) was followed up with a review by an external review pathologist (K Isaacs) conducted in accordance with standard operating procedures. Final diagnoses were made as a consensus between the study pathologist and review pathologist.

Table KIIA 5.5.3/01-4: Organs/tissues collected for pathological examination

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted[a]
Adrenal gland	X	X	X
Aorta	-	X	X
Articular surface (femorotibial joint)	-	X	X
Bone with bone marrow, sternum	-	X	X
Bone marrow smear[(B)]	-	X	-
Brain	X	X	X
Epididymis	X	X	X
Esophagus	-	X	X
Eye (retina, as well as optic nerves)	-	X	X
Gallbladder	-	X	X
Harderian gland	-	X	X
Heart	X	X	X
Intestines (duodenum, jejunum, ileum, cecum, colon, rectum)	-	X	X
Kidney	X	X	X
Lacrymal exorbital gland	-	X	X
Larynx/pharynx	-	X	X
Liver	X	X	X
Lung	-	X	X
Lymph nodes (submaxillary, mesenteric)	-	X	X
Mammary gland	-	X	X
Nasal cavities	-	X	X
Ovary	X	X	X
Pancreas	-	X	X
Parathyroid gland	-	X	X

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted[a]
Pituitary	-	X	X
Prostate	-	X	X
Salivary gland, submaxillary	-	X	X
Sciatic nerve	-	X	X
Skeletal muscle	-	X	X
Seminal vesicle	-	X	X
Skin	-	X	X
Spinal cord, (cervical, lumbar, thoracic)	-	X	X
Spleen	X	X	X
Stomach	-	X	X
Testis	X	X	X
Thymus	-	X	X
Thyroid gland	-	X	X
Tongue	-	X	X
Trachea	-	X	X
Urinary bladder	-	X	X
Uterus with cervix	X	X	X
Vagina	-	X	X
Gross lesions	-	X	X

[A] Histopathological analysis of selected tissues and macroscopic lesions was undertaken for dosing groups. 'B' – Two bone marrow smears from the femur were prepared for each animal and one was stained by the May-Grűnwald-Giemsa method, but was not examined for any group due to the absence of abnormal bone marrow histology findings. The other smear was stored for potential future analysis. Data excerpted from page 30 of the study report.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

There were no observable test substance related clinical signs.

An approx. 2-fold increased incidence (8.3% incidence) and duration of skin lesions was seen in males at 300 and 1500 ppm relative to controls (3.3% incidence), and an increased incidence of alopecia (56.7% incidence) was seen in males at 1500 ppm relative to incidences in all other groups including controls (41.7% incidence). The incidences of skin lesions were 2, 2, 5, 5 and of alopecia were 25, 25, 24, 34 (number animals out of group sizes of 60 animals/group) in the 0, 70, 300 and 1500 ppm dose groups, respectively. Both findings were not statistically significant (*i.e.*, p was >0.05), and on this basis together with the absence of dose dependency, the study authors regarded the findings to be unrelated to the test substance. The evaluator agrees with this conclusion.

2. Mortality

A total of 66 animals were found dead or were humanely sacrificed during the study (Table KIIA 5.5.3/01-5). The incidence of unscheduled mortality was unaffected by the test substance, with ranges of 10 to 22% in males and 10 to 20% in females for all groups including controls. Two instances of premature mortality in females, one at 70 ppm, and one at 1500 ppm, were due to accidents during handling and/or sampling, but are included in the mortality rates below.

Of premature mortalities due to humane sacrifice, at 70 ppm one male (T1M2022) was sacrificed for humane reasons due to an anogenital wound, enlarged spleen and subcutis mass near the preputial gland.

At 300 ppm one male (T3M2259) was sacrificed on study day 329 with a distended urinary bladder, and one female (T3F2319) at this dose was subjected to humane sacrifice at study day 233 with cutaneous alopecia and an enlarged axillary lymph node. One male (T4M2386) at 1000 ppm was humanely sacrificed on study day 295 with an abscess near the preputial gland.

Overall, the frequency of unscheduled deaths was unaffected by treatment, and no deaths were attributed by the study pathologist to exposure to the test substance.

Table KIIA 5.5.3/01-5: Premature (<78 weeks) mortality incidence in carcinogenicity group animals [N=50]

	Dose (ppm)							
	M				F			
	0	70	300	1500	0	70	300	1500
Number of deaths	11	5	9	8	8	10	5	7
% incidence	***22***	***10***	***18***	***16***	***16***	***20***	***10***	***14***

No premature mortality findings were statistically significant. Data excerpted from page 34 of the study report.

B. BODY WEIGHT AND BODY WEIGHT GAIN

Bodyweights and bodyweight gains at 70 ppm were unaffected by treatment (Table KIIA 5.5.3/01-6).

At 300 ppm, the overall mean body weight during the study and at study termination was similar to controls; minor variations were considered to be within inter-animal variation. Minor to moderate decreases in mean body weight gain were noted, with statistical significance noted in both sexes on a few occasions during the treatment period. Overall, bodyweight gain over the treatment period was decreased by 6% in males, and 10% in females compared with controls (not statistically significant).

At 1500 ppm, animals gained bodyweight over the treatment period, though a progressive increase in the body weight difference between 1500 ppm animals and controls was observed with study progression, resulting in statistically significant decreases (-6/-7% in males/females) in terminal bodyweights at week 78. However, US EPA secondary reviewer considered the absolute body weight decrease did not approach the magnitude of being adverse. Mean bodyweight gain was decreased at statistically significant levels at the majority of recording intervals in males, and from week 14 onwards in females (Table KIIA 5.5.3/01-6). The overall body weight gain over the study period (days 1–540) was decreased by 19% in males and 13% in females compared with controls.

Table KIIA 5.5.3/01-6: Bodyweight and bodyweight gain

	Dose (ppm)							
	M				F			
	0	70	300	1500	0	70	300	1500
Bodyweight (g)								
Initial BW (D 1)	20.6±1.1	20.6±1.1	20.6±1.0	20.6±1.0	17.2±1.1	17.0±0.9	16.9±1.0	16.8±0.9
BW Wk 2 (D 8)	21.3±1.1	21.4±1.1	21.0±1.0	21.0±1.0	17.6±1.0	**17.1±1.2*** **(-3%)**	**17.1±1.0*** **(-3%)**	**17.1±1.1*** **(-3%)**
BW Wk 14 (D 92)	26.4±1.3	26.3±1.4	26.5±1.1	26.1±0.9	22.0±1.3	22.3±1.2	22.2±1.0	22.0±1.0
BW Wk 26 (D 176)	28.6±1.4	28.2±1.4	**27.7±1.3**** **(-3%)**	**26.9±1.3**** **(-6%)**	23.7±1.4	24.1±1.5	23.3±1.3	**23.1±1.1*** **(-3%)**
BW Wk 54 (D 372)	30.2±1.6	30.3±1.7	30.3±1.6	**28.9±1.7**** **(-4%)**	25.8±2.6	25.9±2.4	25.5±1.8	**24.8±1.7 (-4%)**
Final BW Wk 78 (D 540)	**31.2±1.5**	**30.9±1.6**	**30.7±1.8**	**29.4±1.6**** **(-6%)**	**28.5±3.2**	**28.4±2.9**	**27.1±2.8 (-5%)**	**26.6±2.1**** **(-7%)**
Bodyweight gain (g)								
BWG Wks 1-2 (D 1 to 8)	0.67±0.49	0.71±0.44	**0.38±0.48**** **(-43%)**	**0.40±0.42**** **(-40%)**	0.36±0.61	0.14±0.63 (-61%)	0.13±0.51 (-64%)	0.27±0.61 (-25%)
BWG Wks 1-14 (D 1 to 92)	5.77±0.88	5.65±1.03	5.90±0.72	5.5±0.77 (-5%)	4.76±1.00	5.32±1.02** (+12%)	5.31±0.89** (+12%)	5.15±0.71* (+8%)
BWG Wks 14-26 (D 92 to 176)	2.20±0.78	1.889±0.94	**1.20±0.89**** **(-45%)**	**0.81±1.08**** **(-63%)**	1.73±0.67	1.85±0.83 (+7%)	**1.09±0.70**** **(-37%)**	**1.09±0.65**** **(-37%)**
BWG Wks 26-54 (D 176 to 372)	1.80±0.85	2.25±0.94	2.55±0.85** (+42%)	2.01±1.08 (+12%)	2.05±1.80	1.93±1.21 (-6%)	2.20±1.17 (+7%)	1.71±1.02 (-17%)
BWG Wks 54-78 (D 372 to 540)	0.91±1.06	0.64±0.92	**0.31±1.13*** **(-66%)**	**0.22±0.81**** **(-76%)**	2.35±2.12	2.32±1.88	**1.45±1.83*** **(-38%)**	**1.58±1.07*** **(-33%)**
Overall BWG (D 1 to 540)	**10.81±1.54**	**10.4±1.27**	**10.17±1.49 (-6%)**	**8.74±1.22**** **(-19%)**	**11.17±3.00**	**11.49±2.42 (+3%)**	**10.13±2.59 (-9%)**	**9.69±1.71*** **(-13%)**

'BWG' - denotes 'bodyweight gain'. 'Wk' - denotes 'week'. '*' – denotes statistical significance at the * $p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from pages 35 and 219–253 of the study report.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

The lower bodyweight gains observed at 1500 ppm were accompanied by comparatively smaller decreases in food consumption in both sexes during study weeks 1 and 2 (3 to 4% lower, no statistical significance), and throughout the study in females only (-2–3% vs controls, no statistical significance). However, mean food consumption was similar to controls when considered across the treatment period (Table KIIA 5.5.3/01-7).

Estimates of food use efficiency were not provided, but given that food consumption in males was unaffected after study week 1, and was only decreased by up to 3% in females over the course of the study, the evaluator considers that decreased bodyweight gains in both sexes at 2500 ppm were at least partially due to decreased food use efficiency.

Table KIIA 5.5.3/01-7: Group mean food consumption (g/animal/day)

	Dose (ppm)							
	M				F			
	0	70	300	1500	0	70	300	1500
Wk 1 to 13	4.13	4.16	4.14	4.16	4.27	4.36	4.26	4.16 **(-3%)**
Wk 1 to 53	4.10	4.13	4.11	4.11	4.24	4.31	4.24	4.14 **(-2%)**
Wk 1 to 77	4.10	4.15	4.12	4.13	4.29	4.35	4.29	4.17 **(-3%)**

No changes were statistically significant Data excerpted from pages 36 of the study report.

D. CLINICAL PATHOLOGY (haematology)

There were some instances of statistically significant decreases in mean corpuscular volume and/or mean corpuscular haemoglobin at 1500 ppm, but the changes from control were slight (less than 3%) and were not accompanied by any changes in red blood cell counts, haemoglobin levels or haematocrit values. In addition, mean platelet levels were elevated in 1500 ppm males compared to controls at both week 5 (13%) and week 78 (12%), but statistical significance was not attained. Elevated platelet levels were seen in short-term rat and dog studies conducted with BYI 02960. Hematology investigations were not conducted in short-term mouse studies. The finding of increased platelets is considered to be treatment-related and potentially adverse.

F. PLASMA CONCENTRATION

Analyses of test substance in blood plasma indicated that overall, the concentration of test substance in plasma scaled in an approximately linear manner with increasing dietary dose at both 52 and 78 week time points (Table KIIA 5.5.3/01-8). There were minor sex differences in test substance concentrations at both time points, with males presenting with slightly elevated concentrations compared with females. While a slight elevation of week 78 blood plasma concentrations (up to +16%) was noted compared with 52 week samples, the differences observed are likely due to inter-animal variation and not considered evidence of test substance accumulation.

Table KIIA 5.5.3/01-8. Individual & group mean plasma concentrations of BYI02960 (but not metabolites) (mg/L±SD)

N=5 (or 2 for control)	Dose (ppm)			
	0	**70**	**300**	**1500**
Week 52				
Males				
Animal Nº's	**T1M22074, 2078**	**T2M2192, 2195, 2196, 2197, 2198**	**T3M2312, 2315, 2316, 2317, 2318**	**T4M2431, 2434, 2436, 2437, 2438**
	<LLOQ	1.43	6.13	24.3
	<LLOQ	1.39	7.34	33.5
	NA	1.28	5.44	24.1
		1.02	4.95	30.7
		1.33	6.38	24.3
Group Mean	<LLOQ	1.29±0.16	6.05±0.92	27.4±4.42
Fold-increase from 70 ppm		***-***	***4.7***	***21.2***
Females				
Animal Nº's	**NA**	**T2F2246, 2247, 2248, 2253, 2255**	**T3F2373, 2374, 2376, 2377, 2378**	**T4F2493, 2495, 2496, 2497, 2498**
	NA	1.14	5.59	26.9
		1.06	2.98	17.9
		1.06	4.22	29.8
		1.20	3.00	24.7
		0.245	4.24	24.6
Group Mean	NA	0.94±0.39	4.01±1.08	24.8±4.39
Fold-increase from 70 ppm		***-***	***4.3***	***26.4***
% change from males		***-27%***	***-33%***	***-9.5%***
Week 78				
Males				
Animal Nº's [(B)]	**T1M22074, 2078**	**T2M2193, 2194, 2196, 2197, 2198**	**T3M2311, 2312, 2315, 2316, 2318**	**T4M2429, 2430, 2431, 2437, 2438**
	<LLOQ	1.31	5.31	29.8
	<LLOQ	1.46	7.75	30.3
	NA	1.57	6.06	27.8
		1.59	5.66	32.2
		1.34	8.14	31.4
Group Mean	<LLOQ	1.45±0.13	6.58±1.28	30.3±1.68
Fold-increase from 70 ppm		***-***	***4.5***	***20.9***
Fold-increase from wk 52 (and %)		***1.12 (±12%)***	***1.09 (±9%)***	***1.11 (±11%)***
Females				
Animal Nº's	**NA**	**T2F2245, 2247, 2252, 2253, 2255**	**T3F2374, 2375, 2376, 2377, 2378**	**T4F2493, 2494, 2495, 2497, 2498**
	NA	1.14	3.83	25.4
		0.693	4.54	34.5
		1.24	4.03	26.2
		0.930	2.30	28.8
		1.15	4.79	28.8
Group Mean	<LLOQ	1.03±0.22	3.90±0.97	28.7±3.56
Fold-increase from 70 ppm		***-***	***3.8***	***27.9***
Fold-increase from week 52 (and %)		***1.10 (±10%)***	***0.97 (-3%)***	***1.16 (±16%)***
% change from males		***-29%***	***-40.7%***	***-5.3%***

Lower limit of quantification (LLOQ) = 0.025 mg/L. Animal numbers are presented relative to their order of appearance in the table (e.g., results for control male animal 22074 is presented first in that column). Data excerpted from pages 2797 and 2798 of the study report, except for fold-increases from 70 ppm values and fold increases from males and from weeks 52 to 78, which were calculated by the evaluator using Microsoft Excel.

G. SACRIFICE AND PATHOLOGY

1. Organ weight

The majority of organ weights were unaffected by treatment. Organ weight changes were confined to decreased kidney weights in males at 1500 ppm, and increased liver weights in both sexes at 1500 ppm (Table KIIA 5.5.3/01-9).

Absolute kidney weights were decreased (-13%) in males at statistically significant levels compared with controls at both the interim and terminal sacrifice at 1500 ppm. Relative-to-bodyweight kidney weights (-8–10% at both sacrifice intervals; statistically significant at terminal sacrifice only), and relative-to-brain kidney weights (-14–15%, statistically significant at both sacrifice intervals) were also recorded.

Statistically significant increases in absolute (9%, $p \leq 0.01$), relative-to-bodyweight (14%, $p \leq 0.01$) and relative-to-brain weight (9%, $p \leq 0.01$) liver weights were observed in 1500 ppm males at terminal sacrifice (week 78). Statistically significant increases in the relative-to-bodyweight liver weights in females at 1500 ppm on study week 78 were also noted (8%; $p \leq 0.01$) relative to controls, though absolute and relative to brain weight values were similar to controls.

Additionally, increased relative brain weights were seen in both sexes at scheduled sacrifice (5/7% increases in M/F, $p \leq 0.01$), and in males at interim (9%, $p \leq 0.05$) sacrifice. However, the increases were accounted for by decreased bodyweight relative to controls, with absolute brain weights remaining unaffected.

Overall, in the absence of additional clinical pathology data, and noting the histopathological changes observed in the kidney and liver (see Section G.2 below), the evaluator regards the decrease in kidney weights (absolute and relative to bodyweight and brain weight) at study week 52 and onwards in males at 1500 ppm to be test substance related and potentially toxicologically adverse. However, the increased liver weights at 1500 ppm in males were considered treatment-related and toxicologically adverse.

Table KIIA 5.5.3/01-9. Organ weight findings at scheduled sacrifice on study weeks 52 or 78

Organ parameter	M				F			
	Dose (ppm)							
	0	70	300	1500	0	70	300	1500
Study week 52 sacrifice								
Absolute kidney weight (g)	0.4867 ±0.0337	0.4810 ±0.0565 **(-1%)**	0.4749 ±0.0342 **(-2%)**	0.4231 ±0.0471 **(-13%)***	0.3565 ±0.0429	0.3774 ±0.0364 **(+6%)**	0.3523 ±0.0525 **(-1%)**	0.3491 ±0.0246 **(-2%)**
Kidney to bw (%)	1.7840 ±0.1259	1.7809 ±0.1213 **(0%)**	1.7956 ±0.1255 **(+1%)**	1.6500 ±0.1433 **(-8%)**	1.6861 ±0.0918	1.6436 ±0.0924 **(-3%)**	1.6479 ±0.1337 **(-2%)**	1.6386 ±0.0935 **(-3%)**
Kidney to brain weight (%)	108.4771 ±5.7003	107.5704 ±13.7494 **(-1%)**	106.8380 ±8.7855 **(-2%)**	92.3500 ±9.0416 **(-15%)****	76.6298 ±6.3735	81.1747 ±7.7034 **(+6%)**	76.1428 ±9.2563 **(-1%)**	74.6949 ±5.3886 **(-3%)**
Study week 78 sacrifice								
Absolute kidney weight (g)	0.4959 ±0.0482	0.5148 ±0.0489 **(+4%)**	0.4932 ±0.0505 **(-1%)**	0.4290 ±0.0384 **(-13%)****	0.4424 ±0.0497	0.4533 ±0.0832 **(+2%)**	0.4381 ±0.0455 **(-1%)**	0.4187 ±0.0420 **(-5%)**
Kidney to bw (%)	1.8295 ±0.1406	1.9004 ±0.1321 **(+4%)**	1.8073 ±0.1583 **(-1%)**	1.6538 ±0.0966 **(-10%)*****	1.7650 ±0.1179	1.8233 ±0.3017 **(+3%)**	1.7957 ±0.1407 **(+2%)**	1.7800 ±0.1246 **(+1%)**
Kidney to brain weight (%)	109.5244 ±10.1927	113.2376 ±10.4639 **(+3%)**	108.8135 ±9.7886 **(-1%)**	94.2427 ±7.5710 **(-14%)****	93.9341 ±8.6909	95.1333 ±17.5359 **(+1%)**	92.2409 ±8.8349 **(-2%)**	88.5742 ±7.4842 **(-6%)**
Absolute liver weight (g)	1.128 ±0.0784	1.128 ±0.0846 **(0%)**	1.137 ±0.0727 **(+1%)**	1.233 ±0.0861 **(+9%)****	1.285 ±0.1749	1.287 ±0.1751 **(0%)**	1.220 ±0.2035 **(-5%)**	1.294 ±0.1899 **(+1%)**
Liver to body weight ratio (%)	4.166 ±0.2381	4.171 ±0.2616 **(0%)**	4.172 ±0.2388 **(0%)**	4.759 ±0.2743 **(+14%)****	5.110 ±0.3489	5.167 ±0.4445 **(+1%)**	4.986 ±0.6718 **(-2%)**	5.509 ±0.8036 **(+8%)****
Liver to brain weight ratio (%)	249.155 ±16.3057	248.368 ±19.3286 **(0%)**	251.190 ±15.1611 **(+1%)**	270.814 ±16.0455 **(+9%)****	272.958 ±34.0733	270.002 ±33.9804 **(-1%)**	257.339 ±44.1777 **(-6%)**	273.926 ±39.0896 **(0%)**

'*' Denotes statistical significance at the *p≤0.05, **p≤0.01, or ***p≤0.001 level. Data excerpted from pages 38 and 39 of the study report.

2. Gross pathology and histopathology

At the interim sacrifice (52 weeks), gross necropsy findings in interim sacrifice animals were unremarkable. No histopathology was conducted at the interim sacrifice.

At the terminal sacrifice (78 weeks), abnormal gross necropsy findings in males at 1500 ppm were confined to a statistically significant increase (5/42 incidence; p≤0.05) in atrophic/small kidneys (Table KIIA 5.5.3/01-10). Additional treatment-related histopathological changes in the kidney included decreased incidence and extent of bilateral basophilic tubules and focal cortical mineralisation, with the changes identified at statistically significant levels (all p≤0.01). The primary evaluator notes that the described kidney histopathology changes were decreased in frequency for 1500 ppm animals compared with controls, and the effects may not be e toxicologically adverse; the US EPA secondary reviewer agrees with this assessment. However, the PMRA secondary reviewer notes that in the absence of clinical pathology and urinalysis, these findings of decreased incidence of bilateral basophilic tubules and focal cortical mineralisation cannot be dismissed as being non-adverse. In addition, in the 90-day mouse study, a decreased incidence of diffuse cortico-epithelial vacuolation was noted in high dose males and was

considered adverse based on the associated changes in kidney weight and clinical chemistry parameters.

Treatment-related changes in liver histopathology were also noted. An increased severity of diffuse hepatocellular vacuolation (mainly centrilobular) was seen in males at 300 ppm without statistical significance, and an increased incidence and severity was seen in males at 1500 ppm with statistical significance ($p \leq 0.01$). In females at 1500 ppm, a statistically significantly ($p \leq 0.01$) decreased incidence of diffuse hepatocellular macrovacuolation (mainly periportal) was observed. The study authors regarded the histopathological changes in the liver to be test substance related but not adverse, on the basis of an absence of degenerative changes. While the lack of other endpoint data (e.g. clinical pathology) limits the ability to clarify the adverseness of the histopathological findings, the evaluator considers that decrease in periportal macrovacuolation in 1500 ppm females is unlikely to be toxicologically adverse given the direction of change compared with controls. However, noting that a similar histopathological change was observed in the two-year chronic/carcinogenicity study in rats (Garcin, 2012; SA 08337), the increase in frequency and severity of diffuse hepatocellular vacuolation in 1500 ppm males is likely to be toxicologically adverse and correlated with the increased liver weight changes observed.

There was no test substance related effects on the total incidence or time of onset for neoplastic findings.

Table KIIA 5.5.3/01-10: Necropsy and histological findings at 78-week scheduled sacrifice

	Dose (ppm)							
	M				**F**			
	0	**70**	**300**	**1500**	**0**	**70**	**300**	**1500**
Atrophic/small kidneys (animals killed at terminal sacrifice)								
Total	0/38 **(0%)**	0/45 **(0%)**	0/41 **(0%)**	**5/42*** **(12%)**	0/42 **(0%)**	0/39 **(0%)**	0/45 **(0%)**	0/42 **(0%)**
Basophilic tubules: bilateral								
Minimal	23	23	18	3	3	2	3	1
Slight	4	2	3	0	0	0	0	0
Total	**27**	**25**	**21**	**3****	**3**	**2**	**3**	**1**
Cortical mineralisation: focal								
Minimal	12	19	13	1	1	0	0	0
Moderate	0	0	1	0	0	0	0	0
Total	**12**	**19**	**14**	**1****	**1**	**0**	**0**	**0**
Corticoepithelial vacuolation								
Minimal	3	2	5	19	0	0	0	0
Slight	4	3	7	9	0	0	0	0
Moderate	29	26	31	4	0	0	0	0
Marked	13	14	4	0	0	0	0	0
Total	**49**	**45**	**47**	**32****	**0**	**0**	**0**	**0**
Diffuse hepatocellular macrovacuolation: mainly periportal								
Minimal	0	0	0	0	32	28	28	13
Slight	0	0	0	0	2	1	1	0
Total	**0**	**0**	**0**	**0**	**34**	**29**	**29**	**13****
Diffuse hepatocellular vacuolation : mainly centrilobular								
Minimal	10	16	2	0	0	0	0	0
Slight	16	16	22	5	0	1	0	1

Moderate	2	2	12	31	0	1	0	3
Marked	0	0	0	5	1	0	0	0
Total	**28**	**34**	**36**	**41****	**1**	**2**	**0**	**4**

* denotes statistically significant at the * p≤0.05 or ** p≤0.01 level. Data excerpted from pages 41 and 42 of the study report. Group size n=50 unless specified.

III. CONCLUSION

Under the conditions of this study, BYI02960 produced no adverse effects on survival, clinical signs of toxicity or haematology parameters across all dose levels tested. No treatment-related changes in the frequency of carcinogenicity, or the time of onset of neoplastic effects were observed.

Blood plasma analyses indicated that the concentration of test substance in plasma scaled in an approximately linear manner with increasing dietary dose at both 52 and 78 week time points (Table KIIA 5.5.3/01-8). There were minor sex differences in test substance concentrations at both time points, with males presenting with slightly elevated concentrations compared with females. However, no accumulation of test substance was noted during the study.

At 70 and 300 ppm no adverse effects were observed with administration of BYI 02960.

At 1500 ppm, treatment-related decreased mean body weights (-6%/-7% for M/F) and bodyweight gain (-19%/-13% for M/F) were observed compared with controls at statistically significantly levels. While food consumption in 1500 ppm animals was similar to controls, with only marginal decreases observed in females (-2–3% vs controls), the decreased mean bodyweight and bodyweight gain suggest decreased food use efficiency, and in this context, the changes in bodyweight and bodyweight gain are considered toxicologically adverse. The US EPA secondary reviewer considered the changes in the body weight parameters not approaching a magnitude of being adverse. . In terminal findings at 1500 ppm, increased absolute and relative liver weights in males were observed and correlated to a toxicologically adverse change in liver histopathology (increased frequency and severity of diffuse hepatocellular vacuolation). Increased incidence of small/atrophic kidneys and decreased kidney weights were seen in 1500 ppm males.

Based on the study findings, the NOAEL in was 300 ppm (43/53 mg/kg bw/d for males/females respectively). The LOAEL was 1500 ppm (224/263 mg/kg bw/d for males/females respectively), based on statistically significant increases in absolute and relative liver weights, with correlative liver histopathology (diffuse hepatocellular vacuolation) and decreased kidney weights and alterations in kidney pathology in males (small/atrophic kidneys).

This study is classified as fully reliable (acceptable/guideline) and satisfies guideline requirements for a carcinogenicity toxicity in mice [OPPTS 870.4200; OECD 451 (1981)].

Primary review: Australia
Secondary review: PMRA & US EPA

Report: Garcin JC (2012) BYI 02960 - Chronic toxicity and carcinogenicity study in the Wistar rat by dietary administration. Bayer SAS. Bayer CropScience. 355 rue Dostoïevski. BP 153, 06903 Sophia Antipolic Cedex, France. Laboratory Report No.: SA 08337. 05 March 2012. MRID 48844123. PMRA 2239460.Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N°TXRVP017. Bayer CropScience File No. M-428257-01-1.

Guidelines: OPPTS 870.4300 (1998)
OECD 453 (1981)
Commission Directive 88/302/EEC, Method B.33 (1987)
MAFF 12 Nousan 8147 (2000)

Deviations: All deviations were of a minor nature and did not affect the reliability of any of the findings described in the following evaluation.

GLP: Yes Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a combined chronic/carcinogenicity study (MRID 48844123), Wistar Rj:WI (IOPS HAN) rats in groups of 70/sex/concentration received BYI 02960 (96.2%; 2009-000239) in the diet at concentrations of 0, 80, 400, or 2000 ppm daily for 52 weeks (10/sex/concentration; interim sacrifice) or 104 weeks (60/sex/concentration). Achieved group mean daily intakes over the entire study duration were 0/0, 3.17/4.48, 15.8/22.5, and 80.9/120 mg/kg bw/d for M/F, respectively. Following overnight fasts on study days 369 to 371 for interim sacrifice groups or study days 739 to 753 for carcinogenicity phase groups, all surviving animals were sacrificed by isoflurane inhalation.

Parameters evaluated included clinical signs, bodyweight, bodyweight gain, food consumption, test compound intakes, haematology, clinical chemistry, coagulation, urinalysis, ophthalmology, organ weights, and gross and microscopic pathology. In addition, blood samples were collected from five randomly selected animals from each group on study weeks 52 and 105, and analysed for levels of parent compound in plasma.

There was no evidence of carcinogenic potential for the test substance at the doses tested in this study. The incidence of premature mortality was unaffected by dosing.

During the first year of dosing, clinical signs were confined to the 2000 ppm dose level in the form of increased incidences of soiled fur for both sexes, alopecia (females only), and in males only, external stimuli hyper-reactivity (7% vs 0 to 1.4% in other groups), and handling resistance (approx. 2 to 4-old increased incidence). All clinical signs had resolved by the second year of dosing with the exception of slight increased incidences of alopecia and soiled fur in males at 2000 ppm. In the absence of other clinical signs of toxicity, the increased incidences of alopecia and soiled fur were not considered toxicologically adverse findings.

Statistically significant differences in mean bodyweights were confined to the 2000 ppm groups in both sexes, In males, a peak decrease of -7% body weight ($p \leq 0.01$) at 2000 ppm compared with controls was noted at study week 14, with a final week 106 difference in body weight of -6% between 2000 ppm and control animals (not statistically significant). In females at 2000 ppm, mean bodyweights were 8% lower than controls by study week 14 ($p \leq 0.01$), with peak bodyweight

differences of -17% between 2000 ppm and control animals at week 78 and final bodyweight differences of -13% terminal bodyweight at 2000 ppm compared with controls at study week 106 ($p \leq 0.01$). It is likely that the decreased bodyweight gain may be secondary to the decreased food consumption observed at 2000 ppm in females.

Ophthalmological findings at the one year examination showed an increased incidence of lens opacity in females at 2000 ppm relative to controls (14.3% vs 4.3%). A small consistent increase in serum cholesterol in females at 2000 ppm relative to controls throughout the study was observed, but this change was not considered to be adverse. Plasma concentration analyses after both one and two years of treatment indicated a consistent relationship between increasing test substance administration and plasma levels of the test substance for both sexes, with a sub-linear relationship observed in males and an approximately linear relationship observed in females.

At both the 52-week and 104-week scheduled sacrifices, treatment-related changes in organ weights were confined to increased relative (to bodyweight) liver weight ($p \leq 0.01$ in both sexes) at 2000 ppm that were accompanied by liver gross and histopathological changes, including centrilobular hypertrophy and macrovacuolation in both sexes; eosinophilic/tigroid/mixed foci of hepatocellular alteration in males, and increased brown pigmentation of Kupffer cells, general hepatocellular brown pigmentation and interstitial mononuclear cell infiltrate in females.

Other treatment-related terminal findings reported in the study included lung findings at 104 weeks in 2000 ppm females, comprising of increased incidence of white foci visible at necropsy. Lung histopathology at 2000 ppm identified lesions comprised of foamy macrophages and chronic interstitial and perivascular inflammation. Additionally, increased thyroid colloid alteration in 2000 ppm males, slightly increased frequency of follicular cell hypertrophy at 2000 ppm in both sexes, and increased brown pigmentation of follicular cells in 2000 ppm females were considered treatment-related and toxicologically adverse.

Adverse effects were not found in 400 and 80 ppm test groups.

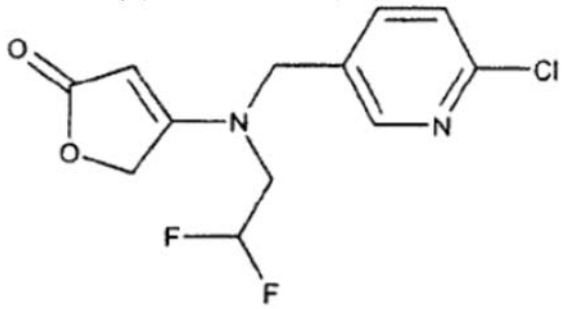
The no-observed adverse-effect level (NOAEL) in this study was 400 ppm (15.8/22.5 mg/kg bw/d for M/F respecitvely). The LOAEL was 2000 ppm (80.9/120 mg/kg bw/d for M/F respectively), based on a range of in-life and terminal changes in both sexes. In-life changes included decreased mean body weight with associated reduction in body weight gain (both sexes), a slightly decreased mean food consumption rate (females only), and a slightly earlier onset of lens opacity (females only) during the first year of treatment. Terminal findings considered included increased relative (to bodyweight) liver weights at study termination (both sexes), which were accompanied by histopathological changes in the liver (centrilobular hypertrophy and macrovacuolation in both sexes; eosinophilic/tigroid/mixed foci of hepatocellular alteration in males; increased brown pigmentation of Kupffer cells, general hepatocellular brown pigmentation and interstitial mononuclear cell infiltrate in females). Additional histopathological changes at 2000 ppm included an increased frequency in a range of effects in the thyroid (colloidal alteration in males, follicular hypertrophy in both sexes) and lungs (fomay macrophages and chronic interstitial and perivascular inflammation). Furthermore, there was an increase in the incidence of mydriasis and pale retina fundus

This study is classified as fully reliable (**acceptable/guideline**) and satisfies guideline requirements for a combined chronic oral toxicity/carcinogenicity study in rats [OPPTS 870.4300; OECD 453 (1981].

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one



   Lot/Batch #: 2009-000239
   Purity: 96.2% (analytical method 02960C01 used)
   Description: Beige powder
   CAS # 951659-40-8
   Stability of test compound: Stored in a closed, light resistant container. Purity was rechecked every six months over the study. Stated to be stable for two years if stored as recommended at 25±5ºC (dates of analysis 16 Jan 2009 and 14 Jan 2011). See Section I.B.3 below for additional stability data.

2. Vehicle and/or positive control: Acetone vehicle used to suspend test compound prior to incorporation into the diet. Diet free of the test substance, but containing acetone, was used for negative controls. No positive control used in the study.

3. Test animals
   Species: Rat
   Strain: Wistar Rj:WI (IOPS HAN)
   Age at dosing: 6 weeks old
   Weight at dosing: 196 to 285 g ($\overline{x}$ 236.5–236.8 g) for males; 136 to 197 g ($\overline{x}$ 166.6–167.6 g) for females
   Source: R. Janvier, Le Genest St Isle, France.
   Acclimation period: 16 days
   Diet: A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*, except at designated time periods including an overnight fast prior to scheduled sacrifice. During the test period, test substance was incorporated into the feed of all animals except negative controls.
   Water: Filtered and softened tap water, *ad libitum*
   Housing: By sex in groups of 5 unless reduced by mortality or isolation in suspended, stainless steel and wire mesh cages.

4. Environmental conditions
   Temperature: 20 to 24°C

Humidity:	40 to 70%
Air changes:	10 to 15/hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion

   10 March 2009 to 22 November 2011

2. Animal assignment and treatment

   Four groups of 60 rats/sex/concentration received concentrations of BYI 02960 at concentrations of 0, 80, 400, or 2000 ppm in feed daily for 104 weeks to examine the carcinogenicity potential of the test substance. After the treatment period, all surviving animals were fasted overnight, then sacrificed and subjected to necropsy. Additional groups of 10 rats/sex/dose received the same concentrations of test substance in feed for 52 weeks and served as interim sacrifice animals for examination of chronic toxicity potential (Table KIIA 5.5.2/01-1). Dose level equivalency expressed as mg/kg bw/d is shown in Table KIIA 5.5.2/01-2.

   The dosing regimen was selected based on the results of the 90-day dietary toxicity study in rats with BYI 02960 (Odin-Feurtet M, 2012; Study No. SA 07294) where a NOAEL was established at 500 ppm in both sexes, based on findings at 2500 ppm (156/186 mg/kg bw/d for M/F), consisting of reversible hepatomegaly associated with minimal to slight centrilobular hepatocellular hypertrophy and increased relative (to bodyweight) liver weight in both sexes, thyroid follicular cell hypertrophy of minimal extent and increased relative (to bodyweight) thyroid weights in males, and 6 to 7% decreases in terminal bodyweights in both sexes relative to controls.

   Prior to randomisation, animals were subjected to ophthalmological and physical examinations, and any animals found to have abnormalities were excluded from the study. Animals were assigned to dose groups by computerised, stratified randomisation so that animals of each sex within the middle range of weight distribution were selected and that weights within each group were within ±20% of the mean on the day of randomisation. A negative control group received untreated diet. Animals that did not meet bodyweight criteria at randomisation were maintained over study week 1 and used as replacements in the instance of premature deaths in the test substance or control groups. At the end of study week 1, 20 males and 20 females from this excess cohort were maintained until the end of the study for health surveillance purposes.

Table KIIA 5.5.2/01-1: Study Design and Compound Intakes

Conc. in diet (ppm)	Mean daily compound intakes mg/kg bw/d		Animal ID No's & time of sacrifice			
			M		F	
	M	F	52 weeks [N=10]	104 weeks [N=60]	52 weeks [N=10]	104 weeks [N=60]
0	0	0	T1M1177 to 1186	T1M1187 to 1246	T1F1247 to 1256	T1F1257 to 1316
80	3.17	4.48	T2M1317 to 1326	T2M1327 to 1386	T2F1387 to 1396	T2F1397 to 1456
400	15.8	22.5	T3M1457 to 1466	T3M1467 to 1526	T3F1527 to 1536	T3F1537 to 1596
2000	80.9	120	T4M1597 to 1606	T4M1607 to 1666	T4F1667 to 1676	T4F1677 to 1736

Day of sacrifice was study week 52 for interim sacrifice subgroup (N=10/sex/dose) and study week 104 for remaining animals (N=60/sex/dose). Doses were not adjusted for compound purity. Data excerpted from pages 29 and 47 of the study report.

Table KIIA 5.5.2/01-2: Test substance intake range over selected intervals

	Dietary concentration (ppm)							
	M				F			
Dose (ppm)	**0**	**80**	**400**	**2000**	**0**	**80**	**400**	**2000**
Weeks 1 to 13	-	5.01	25.1	129	-	6.33	31.7	170
Weeks 1 to 52	-	3.68	18.5	95.1	-	5.08	25.3	136
Weeks 1 to 105	-	3.17	15.8	80.9	-	4.48	22.5	120

Data excerpted from page 47 of the study report.

3. Diet preparation and analysis

BYI 02960 was dissolved into acetone vehicle prior to incorporation into the diet. Diet with acetone free of the test substance was used for controls. Formulations of admixed diet were prepared on a monthly basis, with the exception of the final formulation (F16) which covered the last 10 weeks of the study. When not in use, prepared batches of test substance were stored at room temperature. In total, 16 formulations (F1 to F16) of admixed diet were prepared for each concentration over the 104 week duration of the study.

Homogeneity analysis was undertaken on 10 g samples from each of the surface, middle, and bottom layers of the first loads at 80, 400, and 2000 ppm of F1 (first formulation), and the first loads at 80 and 2000 ppm of F14. For these loads, mean values from this analysis was taken as the mean achieved concentrations.

Additionally, analyses of achieved concentrations was undertaken on a 10 g sample from the surface layer of all loads at all dose levels for F1, F4, F7, F10, F14 and F16, and an additional 10 g sample from each batch was obtained and stored for potential future analysis. For all other formulations, (i.e. F2, F3, F5, F6, F8, F9, F11, F12, F13, and F15), two 10 g samples were obtained for each concentration level and stored for potential future analysis.

Two samples of approximately 40 g were obtained from batches of feed containing 70 ppm and 2500 ppm BYI 02960 used in the carcinogenicity study in mouse (Kennel, 2012). The storage stability of the test substance in the feed when at room temperature for up to 110 days was analysed by HPLC.

The results of the homogeneity analyses (F1 and F14) indicated admixed test substance was found in diet at 85 to 101% of nominal concentrations, while results from concentration analyses indicated that admixed test substance was present at 89 to 100% of nominal

concentrations. Stability analyses on diet samples from Kennel (2012) indicated an approximately 4% degradation of test substance at 70 ppm (final concentration 89% of nominal concentration) and 1% degradation of test substance at 2500 ppm (final concentration 94% of nominal concentration) after 110 days storage at room temperature. Overall, the stability analyses reported that test substance was stable in diet, with admixed test substance found at 89–97% of the nominal concentration. As homogeneity, concentration and stability analyses were within the study author's accepted range of 85 to 115% of nominal concentrations, hence no dose correction factors were required.

4. Statistics

Statistical methods employed in this study are listed in Table KIIA 5.5.2/01-3, and were undertaken using Pristima (version 6.1.0 build 19) with the exception of mean food consumption per day, which was analysed using S.A.S. programs. Statistical significance was assessed to the $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ levels, with the exception of food consumption where only $p \leq 0.05$ and $p \leq 0.01$ were used. For terminal neoplastic and non-neoplastic findings, when the number of lesion-bearing animals was less or equal to two in one group and equal to 0 in the other groups, no statistical test was performed. When the incidences of terminal neoplastic and non-neoplastic findings in 80 and 400 ppm treated groups were equal to 0, only the 2000 ppm treated group was compared to the control group and no trend test was performed. Adjusted survival rates were estimated separately for each sex and treatment group using Kaplan-Meier estimation procedures.

Table KIIA 5.5.2/01-3. Statistics used in data analyses

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant ($p>0.05$)	**If preliminary test is significant ($p \leq 0.05$)**
Terminal bodyweight Clinical chemistry Select haematology parameters (*prothrombin time, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, %neutrophils, %lymphocytes, %reticulocytes*) Quantitative urinalysis *(volume and refractive index)* Organ weight (absolute & relative)	**Bartlett Test**	Analysis of variance followed by 2-sided **Dunnett's test if significant**	**Kruskal-Wallis test** followed by 2-sided **Dunn's test if significant**
Bodyweight and mean food consumption per day parameters Select haematology parameters (*red blood cell count, platelet count, white blood cell count, neutrophil count, lymphocyte count, reticulocyte count)*	**Bartlett Test**	Analysis of variance followed by 2-sided **Dunnett's test if significant**	**Bartlett Test** on log transformed[a] data**;** if significant then **Kruskal-Wallis test** followed by 2-sided **Dunn's test if significant;** Otherwise **ANOVA** followed by **Dunnett test if significant** on transformed data
Urinary pH	**Kruskal-Wallis test**	No further analysis	2-sided **Dunn's test**
Additional analyses in carcinogenicity phase animals only			

Survival	**Cox's and Tarone's tests (two-sided)**
Carcinogenicity incidence, not adjusted for survival	**Cochran-Armitage** method for trend (1-sided) and the **Fisher's exact test** (1–sided)
Carcinogenicity incidence, adjusted for survival	Incidental tumors and non-neoplastic lesions data were analyzed by **logistic regression** of tumours prevalence. Fatal tumors were analysed by the life-table test followed by **Cox's and Tarone's tests.**

[a] Except for haematology parameters, which were square root transformed.

C. METHODS

1. Observations

Animals were observed twice daily for mortality and moribundity (once daily on weekends and public holidays). Animals were observed for clinical signs at least once daily. Animals were examined at least weekly for detailed clinical signs of toxicity including physical examination for palpable masses. The onset, location and dimension of any masses, and/or the nature, onset, severity, duration and recovery of any clinical signs were all recorded. Cages and cage trays were inspected daily for evidence of ill health such as blood or loose faeces.

2. Bodyweights

All animals were weighed at least weekly from the acclimation period through until study week 13, and monthly thereafter until scheduled necropsy.

3. Food consumption, food efficiency and daily intake

Food consumption was recorded twice weekly during study weeks 1 to 6, weekly up to study week 13, then monthly thereafter up to study termination. The mean achieved test substance dosing was defined using the following formula:

$$\text{intake (mg/kg bw/d)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean body weight (g) at the end of the food consumption period}}$$

Food use efficiency estimates were not provided.

4. Ophthalmological examinations

Ophthalmological analyses of each eye were performed by direct ophthalmoscopy, then using the atropinic agent Mydriaticum® (Merck-Sharp and Dohme) and an indirect ophthalmoscope on all animals during acclimation, in control and high dose animals of both sexes at study weeks 52, and in all surviving animals at week 104.

5. Plasma concentration

Blood (non-fasted) was collected from the retro-orbital venous plexus of 5/sex/dose (2 males only for controls) on one morning at 9 am during study weeks 52 and 105. Samples were immediately frozen at -20°C, and test substance concentrations in plasma were determined by HPLC/tandem mass spectroscopy (HPLC-MS/MS) with an LOQ in plasma of 0.025 mg/L BYI 02960. An internal standard for BYI 02960 was used to correlate test substance levels. Examination of recovery rates of a rat plasma sample series loaded with known BYI 02960 concentrations of 0.025 mg/L (98% recovery, N=1), 5.0 mg/L (97 to 102% recovery, N=3), and 50 mg/L (86 to 101% recovery, N=3), produced a mean recovery rate of 96±6.3%, demonstrating appropriate recovery of test substance using the processing methods described.

6. Clinical pathology (hematology, clinical chemistry, coagulation, urinalysis)

During study weeks 14, 26/27, and 52/53 (both interim sacrifice and carcinogenicity phase groups), and during weeks 78 and 104/105 (carcinogenicity phase groups only), animals (10/sex/dose) were fasted overnight in preparation for blood sampling under isoflurane anaesthesia. Blood was collected by puncturing the retro-orbital venous plexus and stored in tubes containing EDTA for hematology (0.5 ml), clot activator for serum chemistry (1.1 mL), and sodium citrate for coagulation (0.9 ml) parameters. On study Day 371 (April 01, 2010), additional blood samples were collected from all groups and stored in tubes containing sodium citrate (0.9 ml) for analysis of coagulation to replace clotted samples. The following clinical pathology parameters were examined:

Hematology and coagulation

The following parameters were measured using an Advia 120 (Simens, Eragny, France), except for blood clotting measurements that were analyzed using an ACL Elite Pro. A blood smear was prepared and stained using May-Grünwald-Giemsa method and examined only in the event of other haematological results being abnormal (blood smears were not examined).

red blood cell count
reticulocyte count
platelet count
white blood cell count
Mean corpuscular (cell) hemoglobin
mean corpuscular (cell) hemoglobin concentration
differential white blood cell count
hemoglobin
hematocrit
mean corpuscular (cell) volume
prothrombin time

Clinical Chemistry

The following parameters were measured using an Advia 1650 (Siemens, Eragny, France). Globulin concentrations and albumin/globulin ratio values were calculated.

aspartate aminotransferase
alanine aminotransferase
alkaline phosphatase
total bilirubin
creatinine
gamma glutamyltransferase
total cholesterol
triglycerides
chloride
glucose
total protein
albumin
calcium
inorganic phosphorus
sodium
urea
potassium

Urinalysis

During study weeks 15, 25/26, and 50/51 (both interim sacrifice and carcinogenicity phase groups), and during weeks 77/78 and 103/104 (carcinogenicity phase groups only), overnight urine samples were collected (approximately 16 hours) from animals (10/sex/dose) under fasting conditions (food and water). Identification of urinary sediment composition after centrifugation (microscopic examination) was undertaken, with the presence of red or white

blood cells, epithelial cells, bacteria, casts and crystals graded. The following parameters were examined:

appearance	ketone
volume	protein
specific gravity/osmolality/refractive index	bilirubin
pH	blood
glucose	urobilinogen
Sediment (microscopic)	

9. Sacrifice and pathology

Following overnight fasts on study days 369 to 371 for interim sacrifice groups or study days 739 to 753 for carcinogenicity phase groups, all surviving animals were sacrificed by exsanguination under isoflurane inhalation. To reduce the effect of differences in dosing duration, equivalent numbers of animals per dosing group were sacrificed across the selected days of termination days. Necropsy included examination of external surfaces, all orifices, all major organs, tissues and body cavities (**Table KIIA 5.5.2/01-4)**. Histopathological analysis in the 52-week interim sacrifice cohort was undertaken for all animals dying before scheduled termination, and in control and high dose groups only at scheduled termination. The liver, lung, kidney, thyroid and gross lesions from intermediate dose group animals were also processed for histopathology. All animals from the carcinogenicity cohort (105 week exposure) were subjected to histopathological examination. Samples were embedded in paraffin wax and subjected to histological analysis using haematoxylin and eosin staining. Samples were fixed by immersion in 10% neutral buffered formalin, with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative.

Initial examinations of macroscopic and microscopic lesions by the study pathologist were followed up with either an in-house peer review (interim sacrifice groups) or external peer review (carcinogenicity groups) conducted in accordance with standard operating procedures. Final diagnoses were made as a consensus between the study pathologist and review pathologist.

Table KIIA 5.5.2/01-4: Organs/tissues collected for pathological examination

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted[a]
Adrenal gland	X	X	X
Aorta	-	X	X
Articular surface (femorotibial joint)	-	X	X
Bone with marrow (sternum)	-	X	X
Bone marrow smear[(B)]	-	X	-
Brain (3 sections)	X	X	X
Epididymis	X	X	X
Esophagus	-	X	X
Eye (with optic nerve)	-	X	X
Harderian gland	-	X	X
Heart	X	X	X
Kidney	X	X	X

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted[a]
Intestines (duodenum, jejunum, ileum, cecum, colon, rectum)	-	X	X
Lacrymal exorbital gland	-	X	-
Larynx/pharynx	-	X	-
Liver	X	X	X
Lung	-	X	X
Lymph nodes (submaxillary, mesenteric)	-	X	X
Mammary gland	-	X	X
Nasal cavities	-	X	-
Ovary	X	X	X
Pancreas	-	X	X
Parathyroid gland	-	X	X
Pituitary	X	X	X
Prostate	X	X	X
Salivary gland, submaxillary	-	X	X
Sciatic nerve	-	X	X
Skeletal muscle	-	X	X
Seminal vesicle	-	X	X
Skin	-	X	X
Spinal cord, (cervical, lumbar, thoracic)	-	X	X
Spleen	X	X	X
Stomach	-	X	X
Testis	X	X	X
Thymus	X	X	X
Thyroid gland	X[A]	X	X
Tongue	-	X	X
Trachea	-	X	X
Urinary bladder	-	X	X
Uterus with cervix	X	X	X
Vagina	-	X	X
Gross lesions	-	X	X

[A] – Stated not to have been recorded in table on page 40 of the study report, but values were provided later in the study.
'B' – Two femoral bone marrow smears from each animal were prepared, of which one was stained by the May-Grünwald-Giemsa method while the other was stored. Smears were not examined as haematology and bone marrow histology findings were unremarkable.

Data excerpted from page 40 of the study report.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

During the first year of dosing, clinical signs in males were confined to the 2000 ppm dose level, with increased incidences of soiled fur (~13% vs 7–9% in other groups), external stimuli hyper-reactivity (7% vs 0–1.4% in other groups), and handling resistance (16% vs 4–8.6% in other groups).

Clinical signs in females over the first year of treatment were confined to 400 ppm and 2000 ppm groups, with soiled fur (8.6% vs 0– 3% in other groups) and alopecia (18.6 and 31% respectively vs. 11.4% in controls).

All clinical signs had resolved by the second year of dosing, with the exception of males at 2000 ppm, which experienced slightly increased incidences of alopecia (6.7% vs 1.7% in controls) and soiled fur (31.7% vs 20.7% in controls).

2. Mortality

The incidence of premature mortality was unaffected by dosing (Table KIIA 5.5.2/01-5), with the exception of a marked decreased incidence in females at 2000 ppm at study termination at 105 weeks, being 23% versus 52% in controls. The decrease in premature (unscheduled) mortality is not considered a toxicologically adverse effect.

Of the animals allocated to the chronic toxicity phase of the study, only five died prematurely before terminal sacrifice at week 52. Four of these animals died during anesthesia, and one male from the low dose group was found dead.

Table KIIA 5.5.2/01-5: Unscheduled mortality in animals allocated to the carcinogenicity phase

Cause of death	Dietary concentration (ppm)							
	M				F			
	0	80	400	2000	0	80	400	2000
Accidental trauma	-	1	-	-	-	1	1	-
Died during anesthesia	-	-	-	-	1	1	1	1
Killed for humane reason	16	13	16	7	17	18	18	11
Found dead	19	25	14	26	13	10	8	2
Mortality	35 **(58%)**	39 **(65%)**	30 **(50%)**	33 **(55%)**	31 **(52%)**	30 **(50%)**	28 **(47%)**	14 **(23%)**

Data excerpted from page 45 of the study report. Group size n=60/dose.

B. BODY WEIGHT AND BODY WEIGHT GAIN

Overall bodyweights and bodyweight gains were unaffected in 80 ppm and 400 ppm groups relative to controls across the treatment period, apart from a slight statistically significant decrease in bodyweight gain (-6/-12% in males and females respectively) at 400 ppm during the first week of treatment. However, noting that the overall bodyweight and bodyweight gain reported was similar to controls across the overall treatment period, the evaluator considers that the decreased bodyweight gain in the first week at 400 ppm is unlikely to be a toxicologically adverse effect.

Statistically significant differences in bodyweights were confined to the 2000 ppm groups in both sexes (Table KIIA 5.5.2/01-6). In males, a peak decrease of -7% body weight ($p \leq 0.01$) at 2000 ppm compared with controls was noted at study week 14, with a final week 106 difference in body weight of -6% between 2000 ppm and control animals (not statistically significant).

In females at 2000 ppm, mean bodyweights were 8% lower than controls by study week 14 ($p \leq 0.01$), with peak bodyweight differences of -17% between 2000 ppm and control animals at week 78 and final bodyweight differences of -13% terminal bodyweight at 2000 ppm compared with controls at study week 106 ($p \leq 0.01$). It is likely that the decreased bodyweight gain may be secondary to the decreased food consumption observed at 2000 ppm (see Section II.C. below). However, the evaluator considers that the decreased mean body weights and body

weight gains observed at 2000 ppm in both sexes to be treatment-related effects of toxicological significance.

Table KIIA 5.5.2/01-6: Mean±SD bodyweight (g) and bodyweight gains (g)

Interval	Dietary concentration (ppm)							
	M				F			
	0	80	400	2000	0	80	400	2000
Initial BW (D 1)	**237±15**	**237±16**	**237±15**	**237±16**	**167±12**	**168±11**	**168±11**	**167±11**
BW Wk 2 (D 8)	296±21	295±21	293±19	280±19** (-5%)	192±14	191±12	190±16	181±12** (-6%)
BW Wk 14 (D 92)	544±49	537±56	540±51	506±46** (-7%)	290±26	288±22	289±28	267±19** (-8%)
BW Wk 26 (D 176)	623±59	612±65	615±60	585±54** (-6%)	319±30	313±25	316±34	289±21*** (-9%)
BW Wk 54 (D 372)	714±71	699±76	712±73	680±69* (-5%)	364±51	354±43	355±53	317±33** (-13%)
BW Wk 78 (D 540)	749±90	730±81	752±88	721±84 (-4%)	421±84	430±70	417±81	349±52** (-17%)
BW Wk 106 (D 736)	**653±80**	**623±76**	**655±93**	**616±72 (-6%)**	**421±59**	**437±71**	**444±92**	**364±61** (-13%)**
BWG Wks 1-2 (D's 1 to 8)	59±9	58±8 (-2%)	56±7* (-5%)	43±7** (-27%)	25±6	23±6 (-8%)	22±7* (-12%)	15±6** (-40%)
BWG Wks 1-14 (D's 1 to 92)	308±44	301±48 (-2%)	303±43 (-2%)	269±39** (-13%)	122±19	120±15 (-2%)	122±21 (0%)	100±12** (-18%)
BWG Wks 14-26 (D's 92 to 176)	78±22	75±19 (-4%)	75±19 (-4%)	80±21 (+3%)	29±12	26±9 (-10%)	27±14 (-7%)	22±10** (-24%)
BWG Wks 26-54 (D's 176 to 372)	89±29	90±30 (+1%)	95±29 (+7%)	93±32 (+4%)	48±28	39±28 (-19%)	37±23 (-23%)	28±16*** (-42%)
BWG Wks 54-78 (D's 372 to 540)	31±50	42±35 (+35%)	39±35 (+26%)	39±36 (+26%)	59±43	76±42 (+29%)	62±41 (+5%)	34±29** (-42%)
BWG Wks 78-106 (D's 540 to 736)	-91±73	-84±62 (nc)	-82±51 (nc)	-113±74 (nc)	39±40	21±41 (-46%)	35±41 (-10%)	15±34* (-62%)
Overall BWG Wks 1-106 (D's 1 to 736)	**416±79**	**392±75(-6%)**	**420±91 (+1%)**	**379±69 (-9%)**	**256±57**	**271±70 (+6%)**	**275±88 (+7%)**	**198±57*** (-23%)**

* Denotes statistically significantly different from control at the *p≤0.05, ** p≤0.01, or ***p≤0.001 level. 'nc' – denotes 'not calculated'. Values in parentheses represent the percent change from control. Data excerpted from pages 46 and 341-403 of the study report.

C. FOOD CONSUMPTION

Food consumption in treated male animals was similar to controls across all dose levels, except during the first few days of dosing (days 1 to 5) during which an 11% (p<0.01%) decrease in mean food consumption was observed in 2000 ppm males when compared to controls (data not shown). Statistically significant reductions of 4% to 7% in mean food consumption were noted sporadically thereafter in 2000 ppm males when compared to controls. However, overall food consumption in males was generally similar across all dose groups (Table 5.5.2/01-7).

In female animals, a statistically significant decrease of 11% (p<0.01) in mean food consumption at 2000 ppm was noted during the first few days of dosing (days 1-5; data not shown) when compared to controls. A consistent slight decrease in mean food consumption was observed at 2000 ppm from week 27 onwards. Noting the decreased body weight gain across the treatment period in 2000 ppm females, it is likely that the decreased food consumption is also a treatment-related effect. Food consumption at lower doses in females was unaffected by treatment.

Table KIIA 5.5.2/01-7: Group mean absolute food consumption (g/animal/day)

	Dietary concentration (ppm)							
	M				F			
	0	80	400	2000	0	80	400	2000
Week 1 to 13	27.2	27.0	27.1	26.2	19.8	19.8	19.7	19.8
Week 14 to 26	25.5	25.4	25.8	25.0	18.7	18.5	18.6	18.7
Week 27 to 52	25.9	25.2	25.9	25.6	19.5	18.8	18.7	18.1 (-7%)
Week 53 to 78	24.1	24.2	24.3	24.1	19.5	19.5	19.5	17.7 (-9%)
Week 79 to 104	22.7	22.2	22.6	21.9	19.9	20.7	20.7	18.5 (-7%)

Data excerpted from page 47 of the study report.

D. OPHTHALMOLOGICAL EXAMINATIONS

Ophthalmological findings at the one year examination (Table KIIA 5.5.2/01-8) were confined to an increased incidence of lens opacity in females at 2000 ppm relative to controls (14.3% vs 4.3%; eyes were not examined in intermediate dose groups). At the end of the second year of treatment, the lens opacity frequency increased to 96% incidence in females at 2000 ppm vs a 50% incidence in controls. However, the incidence of this effect in females after two years was only slightly higher than the mean incidence (92%) for historical controls, and was within the historical control range which extended up to 100% (Table KIIA 5.5.2/01-9). While the overall frequency of lens opacity at 2 years is within historical controls in females at 2000 ppm and the concurrent control incidence was low relative to the historical control values, the evaluator considers that with the available data, the observation of a slightly increased frequency in the early onset of lens opacity during the first year of treatment in 2000 ppm females should be considered a treatment-related effect.

Other ophthalmology findings after two years of treatment were unremarkable. Retinal fundus discolouration was present in one animal each of all male dosing groups but not in controls, and was present in three (6.3%) females at 2000 ppm but not in any other female group including controls. Iridial mydriasis was observed at low incidence (2–3/dose group) in all male groups except 2000 ppm, and was only present in 2000 ppm females at a similar incidence (4 total). The evaluator considers that the retinal and iridial effects are likely unrelated to treatment in this case, noting the frequency of the effects and the distribution across dose groups. **Table KIIA 5.5.2/01-8: Ophthalmological findings at the end of study year 1**

	Dietary concentration (ppm)			
	M		F	
	0	2000	0	2000
[N]	**68**	**70**	**69**	**70**
Lens opacity	2 (2.9%)	2 (2.9%)	3 (4.3%)	10 (14.3%)

Data excerpted from page 48 of the study report.

Table KIIA 5.5.2/01-9: Ophthalmological findings at the end of study year 2

	Dietary concentration (ppm)							
	M				F			
	0	80	400	2000	0	80	400	2000
[N]	**26**	**24**	**31**	**31**	**30**	**32**	**33**	**48**

Iris mydriasis	3 (11.5%)	2 (8.3%)	3 (9.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (8.3%)
Lens opacity	23 (88%)	22 (92%)	29 (94%)	29 (94%)	15 (50%)	22 (69%)	27 (82%)	46 (96%)
HCD for lens opacity – Mean % Min-Max)	ND				92.0% (81.4 to 100.0)			
Retina, fundus abnormal colour, pale	0 (0%)	1 (4.2%)	1 (3.2%)	1 (3.2%)	0 (0%)	0 (0%)	0 (0%)	3 (6.3%)

'HCD' - denotes 'historical control data'. 'ND' – denotes no data was provided. Data excerpted from page 48 of the study report.

E. CLINICAL PATHOLOGY

1. Hematology

Statistically significant haematology findings were confined to 2000 ppm in both sexes over the first 18 months of treatment (Table KIIA 5.5.2/01-10): no treatment-related changes were observed at ≤400 ppm.

In females at 2000 ppm (relative to controls), MCV was statistically significantly ($p \leq 0.05$) decreased by 2–3% during the first year of treatment. MCH was decreased by 3–5% ($p \leq 0.05$) at between 3 and 18 months, and platelets were increased by 17% ($p \leq 0.05$) at 3- and 6-month sampling times. Haemoglobin was only affected at study days 180 and 358 and not at any other time point, being 4% lower at both time points ($p \leq 0.01$ on study day 180, and $p \leq 0.05$ on study day 358). This effect coincided with 3–4% decreases in haematocrit at these time points ($p \leq 0.05$ study day 180, and $p \leq 0.01$ on study day 358). Additional changes noted at 2000 ppm were intermittent statistically significant increases in leukocytes (first 12 months in both sexes, extending to 24 months in males) and lymphocytes (first 18 months of the study in both sexes). An additional finding was decreased prothrombin time in males at 2000 ppm on study day 180 (3.6% decrease, $p \leq 0.05$; data not shown), but this finding was not seen at any other timepoint and was not seen in females.

At 24 months, a statistically significant ($p < 0.05$) increase in the absolute neutrophil count of 143% was observed in 2000 ppm males when compared to controls, as were non-statistically significant increases in absolute lymphocyte (23%) and reticulocyte (74%) counts. However, it was noted that these three parameters, along with the total white blood cell counts, had high degree of variability in 2000 ppm males.

The primary reviewer notes that overall, the magnitude of the described changes in red blood cell parameters (i.e., mean cell volume, mean corpuscular haemoglobin, haematocrit and hemoglobin levels) was small; there was a lack of a consistent response in both sexes, and there was an absence of a consistent temporal relationship in the observed effects. Taken together, this suggests that the observed red blood cell parameters are unlikely to be toxicologically adverse.

Table KIIA 5.5.2/01-10: Haematology findings

Parameter	**Dose (ppm)**							
	M				**F**			
	0	**80**	**400**	**2000**	**0**	**80**	**400**	**2000**
MCV (fL)								
day 95	50.4±1.64	50.3±1.10	51.1±1.35	50.8±1.11	52.4±1.43	52.2±1.44	52.2±1.23	**51.3±0.92*** (-2%)**
day 180	51.1±1.62	50.8±1.56	51.8±1.45	51.3±1.32	53.4±1.34	53.2±1.35	53.5±1.05	**52.3±1.15***

Parameter	Dose (ppm)							
	M				F			
	0	80	400	2000	0	80	400	2000
								(-2%)
day 358	50.7±1.7	50.6±2.70	51.6±1.79	50.8±1.30	52.7±1.28	52.5±2.03	52.4±1.61	**51.1±1.37*** **(-3%)**
MCH (pg)								
day 95	17.06±0.521	17.01±0.515	17.42±0.543	17.18±0.473	18.03±0.420	18.06±0.568	18.11±0.397	**17.57±0.485** (-3%)**
day 180	16.99±0.573	16.99±0.67	17.31±0.56	17.08±0.503	18.16±0.407	18.04±0.641	18.08±0.519	**17.49±0.441** (-4%)**
day 358	16.92±0.55	16.91±0.89	17.24±0.49	16.85±0.44	17.88±0.421	17.83±0.60	17.93±0.573	**17.32±0.536** (-3%)**
day 543	16.97±0.81	17.06±0.78	17.35±0.60	16.94±0.54	17.96±0.508	17.82±1.10	18.22±0.48	**17.05±0.29* (-5%)**
***PLT* (10^9/L)**								
day 95	1118.1±207.4	1111.4±208	1126.1±137.6	1202.8±162.5 (+8%)	1192.8±218.01	1190.8±109.24	1182.6±211.93	**1390.1±166.9* (+17%)**
day 180	1169.5±162.1	1180.4±157.1	1141.1±114.3	1208.4±162.8 (+3%)	1238.8±206.1	1214.9±91.03	1199.9±120.3	**1451.8±155.2** (+17%)**
Hb (g/dL)								
day 180	15.42±0.50	15.42±0.43	15.39±0.63	15.26±0.51	15.14±0.49	15.36±0.47	15.33±0.46	**14.48±0.54** (-4%)**
day 358	15.09±0.601	14.90±1.02	15.32±0.50	14.97±0.65	14.59±0.67	14.86±0.62	15.01±0.64	**14.04±0.55* (-4%)**
Hct (L/L)								
day 180	0.4639±0.0161	0.4613±0.017	0.4604±0.017	0.4578±0.015	0.4453±0.159	0.4537±0.0103	0.4535±0.0146	**0.4323±0.0174* (-3%)**
day 358	0.4521±0.021	0.4463±0.028	0.4590±0.018	0.4512±0.019	0.4296±0.0217	0.4380±0.022	0.4389±0.014	**0.4144±0.017** (-4%)**
WBC (10^9/L)								
day 95	14.79±3.329	14.66±2.774	14.58±3.659	15.67±2.604 (+6%)	8.12±2.061	8.43±1.989	8.84±2.074	**10.64±2.504** (+31%)**
day 180	12.13±2.58	12.39±3.01	12.91±3.60	14.07±1.99 (+16%)	6.33±1.647	7.17±1.601 (+13%)	7.02±1.900 (+11%)	**8.51±2.056** (+34%)**
day 358	10.55±1.711	11.27±2.34 (+7%)	11.73±3.35 (+11%)	**12.91±2.034** (+22%)**	5.77±1.42	6.09±1.51 (+6%)	6.33±2.31 (+10%)	6.44±1.29 (+12%)
day 543	8.92±1.582	10.01±1.397	9.08±1.664	**11.68**±1.923** (+31%)	5.85±1.118	5.56±1.109	5.30±1.160	6.36±1.767 (+9%)
day 728	11.10±0.954	11.10±2.167	11.68±2.211	**18.06**±9.055* (+63%)	7.32±2.293	7.94±1.552	7.00±1.65	6.73±2.015
Lymphocytes (10^9/L)								
day 95	11.30±2.231	11.75±2.483 (+4%)	11.62±3.280 (+3%)	12.51±2.248 (+11%)	6.65±1.689	6.69±1.472	7.19±1.847 (+8%)	**8.90±2.290** (+34%)**
day 180	9.56±2.039	9.60±2.313	10.17±2.935	10.93±1.731 (+14%)	4.84±1.366	5.64±1.358	5.40±1.780	6.85±1.796** (+42%)
day 358	7.80±1.30	8.44±1.840 (+8%)	8.86±2.90 (+14%)	**9.31±1.42** (+19%)**	4.08±1.093	4.27±1.25	4.58±1.98 (+12%)	4.64±1.21 (+14%)
day 543	6.32±1.054	7.25±1.03 (+15%)	6.7±1.30 (+6%)	**8.37±1.18** (+32%)**	3.70±0.66	3.64±0.80	3.52±0.66	4.49±1.33 (+21%)

* Denotes statistically significant at the p * ≤ 0.05 or ** ≤ 0.01 level. Data excerpted from pages 424 to 467 of the study report.

2. Clinical chemistry

Effects on clinical chemistry were also confined to the 2000 ppm dosing group for males and females from study day 95 to 728 (termination), and generally decreased in severity over time (Table KIIA 5.5.2/01-11).

Treatment-related clinical chemistry findings were confined to decreased bilirubin in both sexes and increased serum cholesterol in females. While decreases of up to -40% (males) and -46% (females) were observed in bilirubin, the direction of change (decrease) is not considered a toxicologically adverse effect.

A 29% ($p \leq 0.01$) increase in serum cholesterol in females at 2000 ppm relative to controls was seen at study day 95, and remained at a similar magnitude up to study days 180 (+35%,

p≤0.01) and 358 (+24%, p≤0.01), and lost statistical significance thereafter but remained at study day 728 (21% increase). Cholesterol levels in males were unaffected.

Other reported changes in clinical chemistry (chloride, serum glucose, phosphorous, potassium and albumin) were transient and occurred without dose- or temporal relationships.

The evaluator considers that the changes in cholesterol in females are test substance related, though unlikely to be toxicologically adverse given the nature of the effect and the lack of other treatment-related changes in clinical pathology.

Table KIIA 5.5.2/01-11: Clinical chemistry findings

Study day	Dietary concentration (ppm)							
	M				F			
	0	80	400	2000	0	80	400	2000
Total bilirubin (µmol/L)								
95	1.21±0.510 **'a'**	1.23±0.277 (+2%)	1.10±0.316 (-9%)	**0.72±0.334***** **(-40%)**	2.08±0.651	2.18±0.507 (+5%)	1.75±0.517 (-16%)	**1.13±0.339**** **(-46%)**
180	1.60±0.383	1.64±0.536 (+3%)	1.42±0.378 (-11%)	**1.05±0.254***** **(-34%)**	2.45±0.604	2.56±0.494 (+4%)	**2.00±0.407*** **(-18%)**	**1.34±0.347**** **(-45%)**
358	1.75±0.494	1.77±0.426 (+1%)	1.45±0.405 (-17%)	**1.15±0.560**** **(-34%)**	2.69±0.761	2.80±0.710 (+4%)	2.93±0.945 (+9%)	**1.61±0.448***** **(-40%)**
543	1.66±0.259	1.88±0.767 (+13%)	1.71±0.593 (+3%)	1.17±0.362 (-30%)	2.47±0.917	1.81±0.857 (-27%)	1.88±1.104 (-24%)	1.34±0.613 (-46%)
728	2.11±0.743	1.81±0.729 (-14%)	1.64±0.438 (-22%)	1.63±1.763 (-23%)	2.01±0.711	1.82±0.588 (-9%)	1.95±0.712 (-3%)	**1.24±0.363*** **(-38%)**
Total cholesterol (mmol/L)								
95	1.719±0.678	1.522±0.310 (-11%)	1.536±0.193 (-11%)	1.808±0.354 (+5%)	1.680±0.299	1.687±0.325 (0%)	1.648±0.381 (-2%)	**2.164±0.322**** **(+29%)**
180	1.758±0.416	1.699±0.376 (-3%)	1.628±0.241 (-7%)	2.010±0.421 (+14%)	1.713±0.297	1.790±0.280 (+4%)	1.804±0.315 (+5%)	**2.316±0.406**** **(+35%)**
358	2.286±0.496	2.141±0.391 (-6%)	2.228±0.384 (-3%)	2.452±0.443 (+7%)	1.923±0.367	1.921±0.418 (0%)	2.037±0.388 (+6%)	**2.393±0.323**** **(+24%)**
543	2.520±0.376	2.378±0.497 (-6%)	2.796±0.407 (+11%)	2.777±0.512 (+10%)	2.049±0.432	2.010±0.374 (-2%)	2.182±0.535 (+6%)	2.380±0.500 (+16%)
728	2.826±0.644	2.698±0.802 (-5%)	2.987±0.736 (+6%)	2.769±0.840 (-2%)	1.936±0.342	2.281±0.372 (+18%)	2.136±0.344 (+10%)	2.352±0.631 (+21%)

* Denotes statistically significant at the p * ≤ 0.05, ** ≤ 0.01 level or ***p≤0.001 level. 'a' -: mean and standard deviation were calculated excluding a very high value (149.4µmol/L for animal T1M1177 which was considered to have transient jaundice). % changes in the treated groups were calculated with this new mean value, statistical analyses were applied without exclusion. Data excerpted from pages 50 and 51 of the study report, with the exception of cholesterol data for males, which was excerpted from pages 469 to 512, and per cent changes for these values only were calculated using Microsoft Excel whereas all other percentage changes were calculated by the study author.

3. Urinalysis

Urinalysis findings were unremarkable, with the sole finding being a trend for decreased urinary pH in males at 2000 ppm (statistically significantly different from controls at 4 and 6 months) not considered toxicologically adverse due to observed individual animal variation in the parameter.

F. PLASMA CONCENTRATION

At study week 52 and 105, test substance concentrations in blood expressed as (mg/L±SD BYI 02960) increased proportionally to dose. The relationship between dose level and observed plasma concentrations was approximately linear in females, and sub-linear in males (Table KIIA 5.5.2/01-12). Similar to observations in the 90-day dietary study in rats (Odin-Feurtet, 2012; SA

07294), test substance concentrations in plasma were generally higher in females than in males at most concentrations of test substance in feed. The study authors concluded that the plasma concentration results demonstrated a consistent relationship between increasing test substance administration and plasma levels for both sexes, and the evaluator concurs with this observation.

Table KIIA 5.5.2/01-12: Individual & group mean plasma concentrations of BYI 02960 (mg/L±SD)

N=5 (or 2 for control	Dose (ppm			
	0	**80**	**400**	**2000**
Week 52				
Males				
Animal Nº's	**T1M1245, 1246**	**T2M1382, 1383, 1384, 1385, 1386**	**T3M1522, 1523, 1524, 1525, 1526**	**T4M1662, 1663, 1664, 1665, 1666**
	<LLOQ	1.26	4.79	18.1
	<LLOQ	1.55	5.14	19.8
	NA	1.46	5.64	18.3
		1.23	5.45	20.0
		0.934	4.81	18.0
Group Mean	<LLOQ	1.29±0.24	5.17±0.38	18.8±0.98
Fold-increase from 80 ppm		***1***	***4.0***	***14.6***
Females				
Animal Nº's	**NA**	**T2F1452, 1453, 1454, 1455, 1456**	**T3F1591, 1592, 1594, 1595, 1596**	**T4F1732, 1733, 1734, 1735, 1736**
	NA	1.17	8.67	35.8
		1.30	10.7	38.6
		1.47	6.83	31.3
		1.25	6.08	32.4
		1.62	6.92	30.2
Group Mean	NA	1.36±0.18	7.84±1.86	33.7±3.47
Fold-increase from 80 ppm		***1***	***5.8***	***24.8***
% change from males		***+5%***	***+51%***	***+79%***
Week 105				
Males				
Animal Nº's	**T1M1243, 1246**	**T2M1376, 1377, 1382, 1383, 1386**	**T3M1518, 1519, 1520, 1521, 1523**	**T4M1655, 1657, 1661, 1663, 1664**
	<LLOQ	1.68	8.59	20.2
	<LLOQ	1.48	5.32	28.0
	NA	1.45	7.58	23.1
		1.42	7.21	19.7
		1.52	7.27	16.0
Group Mean	<LLOQ	1.51±0.1	7.19±1.18	21.4+4.47
Fold-increase from 80 ppm		***1***	***4.8***	***14.2***
Fold-increase from wk 52 (and % change)		***1.17 (+17%)***	***1.39 (+39%)***	***1.14 (+14%)***
Females				
Animal Nº's	**NA**	**T2F1447, 145?, 1452, 1453, 1455**	**T3F1583, 1584, 1589, 1594, 1595**	**T4F1732, 1733, 1734, 1735, 1736**
	NA	1.27	9.51	33.1
		0.310	8.57	35.5
		0.920	7.56	26.4
		1.15	6.18	28.3
		1.66	9.19	28.4
Group Mean	<LLOQ	1.06+0.5	8.20+1.35	30.3±3.80
Fold-increase from 80 ppm		***1***	***7.7***	***28.6***
Fold-increase from week 52 (and % change)		***0.78 (-22%)***	***1.05 (+5%)***	***0.90 (-10%)***
% change from males		***-30%***	***+14%***	***+41%***

Lower limit of quantification (LLOQ = 0.025 mg/L). Animal numbers are presented relative to their order of appearance in the table (e.g., results for control male animal 1245 are presented first in that specific column). Data excerpted from pages 6409 and 6410 of the study report, except for fold-increases from 80 ppm values and fold increases from males and from weeks 52 to 105, which were calculated by the evaluator using Microsoft Excel.

G. SACRIFICE AND PATHOLOGY

1. Organ weight

At both the 52 week and 104 week scheduled sacrifices, treatment-related changes in organ weights were confined to an increased relative (to bodyweight) liver weight ($p \leq 0.01$) in both sexes at 2000 ppm (Table KIIA 5.5.2/01-13). While there were some sex differences in absolute and relative (to brain weight) liver weights at both interim and terminal sacrifice, the clear statistically significant increase in relative (to bodyweight) liver weights were considered treatment-related, noting the presence of liver-related histopathological changes (see Section G.2 below).

Table KIIA 5.5.2/01-13: Organ weights

Organ parameter	M				F			
	Dose (ppm							
	0	80	400	2000	0	80	400	2000
52 week interim sacrifice animals								
Absolute liver weight (g)	13.421 ±1.7893	13.222 ±1.5469 (-1%)	13.137 ±1.7780 (-2%)	14.856 ±1.8431 (+11%)	7.854 ±1.3550	7.182 ±1.1995 (-9%)	7.245 ±1.2346 (-8%)	7.222 ±0.4811 (-8%)
Liver to bodyweight ratio (%)	1.993 ±0.1374	1.970 ±0.1408 (-1%)	1.946 ±0.1838 (-2%)	2.285 ±0.1736 **(+15%)****	2.135 ±0.1449	2.194 ±0.2280 (+3%)	2.101 ±0.1524 (-2%)	2.444 ±0.1080 **(+14%)****
Liver to brain weight ratio (%)	587.888 ±70.5766	573.101 ±74.3182 (-3%)	572.059 ±78.7533 (-3%)	670.435 ±80.8317 (+14%)	373.068 ±66.9021	344.168 ±59.4331 (-8%)	364.246 ±72.3178 (-2%)	346.880 ±18.2160 (-7%)
104 week terminal sacrifice animals								
Absolute liver weight (g)	12.401 ±1.9940	12.065 ±1.8556 (-3%)	11.971 ±1.8261 (-3%)	12.915 ±1.4947 (+4%)	8.878 ±1.7052	9.246 ±1.6570 (+4%)	9.406 ±2.1925 (+6%)	8.308 ±1.3457 (-6%)
Liver to bodyweight ratio (%)	1.971 ±0.2142	2.008 ±0.2758 (+2%)	1.941 ±0.1859 (-2%)	2.215 ±0.3085 **(+12%)****	2.242 ±0.2658	2.291 ±0.3077 (+2%)	2.278 ±0.3116 (+2%)	2.458 ±0.2783 **(+10%)****
Liver to brain weight ratio (%)	530.945 ±90.8128	518.885 ±81.6223 (-2%)	514.364 ±79.8667 (-3%)	546.361 ±64.5187 (+3%)	418.821 ±79.1927	440.047 ±79.7220 (+5%)	449.204 ±105.7920 (+7%)	392.895 ±67.0007 (-6%)

** Denotes statistically significant at the $p \leq 0.01$ level. Data excerpted from page 53 of the study report.

2. Gross pathology and histopathology

In animals not surviving to terminal sacrifice at 52 or 104 weeks, no treatment-related gross pathology findings were observed except an increased frequency of white foci in the lungs of 2000 ppm females (6/14 [43%] vs 2/31 [6%] in controls) from the carcinogenicity phase of the study that died prematurely. Histopathological examination indicated a range of lung-related effects (inflammation and foamy macrophages) which were considered treatment-related and toxicologically adverse findings.

Necropsy and histopathology findings in the 52 week interim sacrifice and 104 week scheduled sacrifice groups were confined to the liver and thyroid in both sexes, and the lungs in females.

The thyroid pathology findings were minimal at 52 weeks, comprising of colloid alteration at increasing frequency with dose in males (treatment-related at ≥400 ppm), and occurring at 2000 ppm only in females (Table KIIA 5.5.2/01-14).

Treatment-related liver gross pathology findings at 52 weeks included enlarged and pale liver in males only at 2000 ppm. Histopathology findings at 52 weeks in males included an increase in centrilobular hepatocellular hypertrophy at 400 ppm, and increased incidences of eosinophilic and tigroid foci of hepatocyte alteration, centrilobular hypertrophy and centrilobular hepatocellular macrovacuolation at 2000 ppm. In females, an increased frequency of centrilobular hepatocellular hypertrophy and macrovacuolation was observed at 2000 ppm only.

While a decreased incidence of periportal hepatocellular vacuolation in both sexes was observed, the direction of change (decrease) for the periportal hepatocellular vacuolation was not considered a toxicologically adverse effect. Additionally, in the absence of any increased incidences of foci of alteration or hepatocellular macrovacuolation, the study authors regarded centrilobular hypertrophy in two males (20%) at 400 ppm at 52 weeks sacrifice to not be toxicologically adverse. The evaluator agrees with this conclusion.

At terminal examination (104 weeks), clear treatment-related increases in liver histopathology (centrilobular hypertrophy and macrovacuolation) were observed in both sexes at 2000 ppm. Sex-specific liver histopathology was also noted at 2000 ppm, with increased eosinophilic/tigroid/mixed foci of hepatocellular alteration in males, and increased brown pigmentation of Kupffer cells, general hepatocellular brown pigmentation and interstitial mononuclear cell infiltrate in females (Table KIIA 5.5.2/01-15).

At 400 ppm, minimal centrilobular hepatocellular hypertrophy was observed in six males, which, in the absence of any increased incidences of foci of alteration or hepatocellular macrovacuolation, is considered non-adverse.

Treatment-related lung macropathology in females was comprised of increased incidence of white foci visible at necropsy in 2000 ppm animals (Table KIIA 5.5.2/01-15), with corresponding histopathological changes in the lung tissue of females at this dose identified as increased incidences of focal alveolar foamy macrophages (83% vs 50%; $p \leq 0.01$), focal chronic interstitial inflammation (17% vs 3%; $p \leq 0.01$), and focal perivascular inflammation (60% vs 28%; $p \leq 0.01$). Further information on the possible aetiology of the finding is unclear, and the study author did not provide an explanation but regarded the lesions as test substance related and toxicologically adverse. The evaluator concurs with this analysis in this case. There was also a clear decrease in the incidence of granuloma with cholesterol clefts in the lungs of males at 2000 ppm, as well as a decreasing trend in this finding in females. This decrease is not considered to be toxicologically significant.

Treatment-related thyroid findings at carcinogenicity phase termination included increased colloid alteration in 400 and 2000 ppm males, slightly increased frequency of follicular cell hypertrophy at 2000 ppm in both sexes, and increased brown pigmentation of follicular cells in 2000 ppm females. While thyroid effects at 2000 ppm were considered treatment-related and toxicologically adverse when considered together, the toxicological adverseness of colloid alteration at 400 ppm is uncertain given the lack of other consistent treatment-related findings at 400 ppm, though the effect at 400 ppm should be considered a treatment-related effect.

There was no evidence of a neoplastic or carcinogenic potential for the test substance, with no treatment-related neoplastic changes observed in both interim sacrifice and carcinogenicity phase animals.

Table KIIA 5.5.2/01-14: Necropsy and histological findings in interim sacrifice group animals at 52 weeks

	Dose (ppm)							
	M				F			
	0	**80**	**400**	**2000**	**0**	**80**	**400**	**2000**
Necropsy findings, interim sacrifice group, study week 52								
Hepatomegaly	0/10	0/9	0/9	3/10	1/10	1/9	0/8	0/10
Pale liver colouration	0/10	1/9	0/9	3/10	1/10	1/9	1/8	0/10
Histopathology (n=10)								
Eosinophilic focus(i) of hepatocellular alteration								
Minimal	2	6	3	8	0	0	1	1
Total	**2**	**6**	**3**	**8**	**0**	**0**	**1**	**1**
Tigroid focus(i) of hepatocellular alteration								
Minimal	1	1	0	3	3	2	1	1
Total	**1**	**1**	**0**	**3**	**3**	**2**	**1**	**1**
Hepatocellular hypertrophy: centrilobular								
Minimal	0	0	2	7	0	0	0	5
Slight	0	0	0	3	0	0	0	0
Total	**0**	**0**	**2**	**10**	**0**	0	0	**5**
Hepatocellular macrovacuolation: centrilobular: diffuse								
Minimal	0	2	1	6	0	0	0	2
Slight	0	0	0	4	0	0	0	0
Total	**0**	**2**	**1**	**10**	**0**	**0**	**0**	**2**
Hepatocellular vacuolation: mainly periportal: diffuse								
Minimal	5	6	3	1	6	8	7	4
Slight	1	1	2	0	2	0	3	0
Moderate	0	0	1	0	0	0	0	0
Total	**6**	**7**	**6**	**1**	**8**	**8**	**10**	**4**
Colloid alteration (thyroid)								
Minimal	2	1	6	3	0	0	0	3
Slight	0	1	1	3	0	0	0	0
Moderate	0	1	0	0	0	0	0	0
Marked	0	1	0	0	0	0	0	0
Total	**2**	**4**	**7**	**6**	**0**	**0**	**0**	**3**

No necropsy or histopathology findings at 12 months interim sacrifice met the criteria for statistical analysis. Data excerpted from pages 56 to 57 of the study report.

Table KIIA 5.5.2/01-15: Necropsy and histopathological findings in carcinogenicity phase animals at 104 weeks

	Dose (ppm)							
	M				F			
	0	**80**	**400**	**2000**	**0**	**80**	**400**	**2000**
Necropsy findings, terminal sacrifice group, study week 104 (excluding premature deaths)								
White lung foci								
Total	5/25	4/21	4/30	5/27	2/29	10/30	5/32	21/46
Histopathology – number examined = 60								
Eosinophilic focus(i) of hepatocellular alteration								
Minimal	22	21	19	28	14	9	8	16
Slight	4	6	9	9	2	3	1	6
Moderate	1	0	2	7	0	0	1	1
Marked	0	0	0	1	0	0	0	0
Total	27	27	30	45**	16	12	10	23
Tigroid focus(i) of hepatocellular alteration								
Minimal	29	28	32	42	30	30	24	36
Slight	5	8	7	3	5	7	5	5
Moderate	0	0	0	0	1	0	1	0
Total	34	36	39	45	36	37	30	41
Mixed focus(i) of hepatocellular alteration								
Minimal	0	0	0	3	0	0	0	0
Total	**0**	**0**	**0**	**3**	**0**	**0**	**0**	**0**
Hepatocellular hypertrophy: centrilobular								
Minimal	0	0	6	23	0	0	0	27
Slight	0	0	0	2	0	0	0	1
Total	**0**	**0**	**6***	**25****	**0**	**0**	**0**	**28****
Hepatocellular macrovacuolation: centrilobular: diffuse								
Minimal	3	4	3	18	1	0	0	19
Slight	0	0	1	6	0	1	0	5
Moderate	0	1	0	0	1	0	0	0
Total	3	5	4	24**	2	1	0	24**
Hepatocellular macrovacuolation: periportal: diffuse								
Minimal	5	4	2	2	18	22	12	11
Slight	2	3	1	4	8	11	13	2
Moderate	2	1	1	0	2	3	1	0
Marked	0	0	1	1	0	0	0	0
Total	9	8	5	7	28	36	26	13**
Hepatocellular brown pigment : focal								
Minimal	0	0	0	0	1	0	1	13
Slight	0	0	0	0	0	0	0	3
Total	0	0	0	0	1	0	1	16**
Accumulation of brown pigment in Kupffer cells								
Minimal	8	11	10	9	10	9	14	18
Slight	2	5	8	4	4	4	1	8
Moderate	0	0	0	1	1	1	1	1
Total	10	16	18	14	15	14	16	27*
Focal interstitial mononuclear cell infiltrate (liver)								
Minimal	20	19	21	24	22	28	29	35
Slight	1	0	1	2	1	4	0	1
Moderate	0	0	0	0	1	0	0	0
Total	21	19	22	26	24	32	29	36*
Colloid alteration (thyroid)								
Minimal	14	12	28	18	11	10	10	11
Slight	7	9	9	18	1	3	0	4
Moderate	0	1	1	4	0	1	0	0
Marked	0	0	0	0	1	0	0	0
Total	21	22	38**	40**	13	14	10	15

	Dose (ppm)							
	M				F			
	0	**80**	**400**	**2000**	**0**	**80**	**400**	**2000**
Follicular cell hypertrophy: diffuse (thyroid)								
Minimal	1	0	1	3	0	1	0	3
Total	1	0	1	3	0	1	0	3
Brown pigments: follicular cells (thyroid)								
Minimal	16	10	18	22	8	7	5	17
Slight	1	0	1	1	0	0	0	0
Moderate	0	0	1	0	0	0	0	0
Total	17	10	20	23	8	7	5	17
Alveolar foamy macrophages: focal (lungs)								
Minimal	18	24	22	25	27	18	11	25
Slight	10	12	8	7	2	11	6	18
Moderate	2	4	4	3	1	0	2	6
Marked	0	0	0	0	0	0	0	1
Total	**30**	**40***	**34**	**35**	**30**	**29**	**19***	**50****
Chronic interstitial inflammation: focal (lungs)								
Minimal	1	4	4	3	2	3	5	10
Slight	0	1	0	0	0	0	0	0
Moderate	0	0	1	0	0	0	0	0
Total	**1**	**5**	**5**	**3**	**2**	**3**	**5**	**10***
Perivascular inflammation: focal (lungs)								
Minimal	23	20	30	20	17	22	17	35
Slight	1	3	2	1	0	1	0	1
Total	**24**	**23**	**32**	**21**	**17**	**23**	**17**	**36****
Granuloma with cholesterol clefts: focal (lungs)								
Minimal	**17**	**15**	**13**	**1**	**14**	**7**	**6**	**5**
Slight	**3**	**1**	**2**	**0**	**0**	**1**	**0**	**0**
Total	**20**	**16**	**15**	**1**	**14**	**8**	**6**	**5**

* Denotes statistically significant at the p * ≤ 0.05 or ** ≤ 0.01 level Data excerpted from pages 54 to 60 and 995 & 1007 of the study report.

III. CONCLUSION

Under the conditions of this study, BYI 02960 produced no evidence of carcinogenic potential for the test substance.

The NOAEL was 400 ppm (15.8/22.5 mg/kg bw/d for M/F respectively), LOAEL was 2000 ppm (80.9/120 mg/kg bw/day) based on a range of treatment-related effects included decreased mean body weight with associated reduction in body weight gain (both sexes), a slightly decreased mean food consumption rate (females only), a slightly earlier onset of lens opacity (females only) during the first year of treatment, centrilobular hypertocyte hypertrophy and macrovacuolation in both sexes; eosinophilic/tigroid/ mixed foci of hepatocellular alteration in males; increased brown pigmentation of Kupffer cells, general hepatocellular brown pigmentation, interstitial mononuclear cell infiltrate in females, thyroid effects characterized by colloidal alteration in males, follicular hypertrophy in both sexes, and lungs effects (foamy macrophages and chronic interstitial and perivascular inflammation). Furthermore, there was an increase in the incidence of mydriasis and pale retina fundus

This study is classified as fully reliable (acceptable/guideline) and satisfies guideline requirements for a combined chronic oral toxicity/carcinogenicity study in rats [OPPTS 870.4300; OECD 453 (1981)]

Primary review: Australia
Secondary review: US EPA & PMRA

B.6.4.1/01	***In vitro* genotoxicity—Bacterial Assay for Gene Mutation**
Report:	Herbold, B. (2009a) BYI 02960: Salmonella/Microsome Test: Plate Incorporation and Pre-incubation method. Bayer Schering Pharma AG; Wuppertal, Germany. Report No.: AT05387. Study ID: T8080064. Ref: M-354173-01-02. MRID 48844124. Unpubllished.
Dates of work:	6 February – 29 June 2009
Guidelines:	OPPTS 870.5100 (1998), ECC 2000/32/EC, Annex 4D-B13/14 No., L136 (2000), OECD No. 471 (1998).
GLP/QA:	Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary:

In independent trials of a reverse gene mutation assay, BYI 02960 (96.2% Lot No. 2009-000239) was evaluated for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 with and without an exogenous metabolic activation system (Aroclor-induced rat liver S9) in two phases using the plate incorporation method (Experiment 1) and pre-incubation method (Experiment 2). The dose levels tested were 0, 16, 50, 158, 500, 1581 and 5000 µg/plate. The test substance was administered to the test system as a solution in dimethyl sulfoxide (DMSO).

A slight bacteriotoxic effect was observed at the limit dose of 5000 µg/plate in both trials with and/or without metabolic activation. The positive and vehicle controls fulfilled the requirements for a valid test. In the mutagenicity assay using both test methods, no positive mutagenic response occurred.

Under the conditions of this study, BYI 02960 was tested up to the limit dose (5000 µg/plate) and was negative for mutagenic activity in non-activated and S9-activated test systems.

The study is classified as **reliable (acceptable/guideline)** and satisfies the guideline requirements (OPPTS 870.5100; OECD 471) for *in vitro* mutagenicity (bacterial reverse gene mutation) data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960 technical
   Lot/Batch #: 2009-000239
   Purity: 96.2% (date of analysis 16 January 2009)
   Description: Beige powder
   CAS # 951659-40-8
   Stability of test compound: Stability analysis indicated that the test material was stable in DMSO solvent for 24 hours at 37°C.
2. Control materials
   Solvent control: Dimethyl sulfoxide (DMSO) at 100 µL/plate for all materials (except mitomycin C, in de-ionised water)

Positive control, non-activation:

Positive control	Dose (µg/plate)	Strain(s)
Nitrofuratoin	0.2 or 0.4	TA100
Sodium azide	10 or 20	TA1535
4-nitro-1,2-phenylene diamine	0.5 or 1 (TA98) 10 or 20 (TA1537)	TA98, TA1537
Mitomycin C	0.2 or 0.4	TA102 (plate incorporation only)
Cumene hydroperoxide	50 or 75	TA102 (pre-incubation only)

Positive control, activation:

Positive control	Dose (µg/plate)	Strain
2-Aminoanthracene	3 or 6	All strains

3. Activation: Rat liver S9 from Male Sprague-Dawley rats (Aroclor 1254-induced)
   Source: In-house production (Bayer Schering Pharma)
   Protein content: 24.4 mg/mL
   Characterisation: The metabolic activation ability of the S9 was assayed for its ability to metabolize reference mutagens (not specified), and prior to use in experiments checked for sterility by plating and incubation.

S9 mix composition (per 70 mL solution)

Phosphate buffer (pH 7.4):	100 mM
Glucose-6-phosphate:	179.1 mg
NADP:	315.0 mg
KCl:	246.0 mg (co-factor solution) + 20 % of 0.15 M solution
$MgCl_2$:	162.6 mg
S9:	10% (v/v)

4. Test organisms

Salmonella typhimurium strains TA98, TA100, TA102, TA1535, and TA1537 were properly maintained and were checked for appropriate genetic markers and sensitivity (deep rough, UV).

5. Test concentrations for mutagenicity assay

Exp. 1: (Plate Incorporation)	Concentrations of 0, 16, 50, 158, 500, 1581 and 5000 µg BYI02960/plate were evaluated in triplicate in the presence and absence of S9 activation.
Exp. 2: (Pre-incubation)	Concentrations of 0, 16, 50, 158, 500, 1581 and 5000 µg BYI02960/plate were evaluated in triplicate in the presence and absence of S9 activation.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

17–29 June 2009

2. Main assay methods

This study consisted of 2 independent trials, Experiment 1 (by plate incorporation) and Experiment 2 (Pre-incubation).

In both experiments, negative vehicle control, positive controls and six dose levels of the test substance were plated, with overnight cultures of TA98, TA100, TA102, TA1535 and TA1537 on selective minimal agar in the presence and absence of Aroclor-induced rat liver S9 to evaluate the mutagenic potential of the test substance. All dose levels of test substance, negative vehicle control and positive controls were plated in triplicate. Plates were incubated for 48 hours at 37°C. No untreated control was used in this study.

For Experiment 1 (plate incorporation test), 0.1 mL of test material, 0.1 mL bacterial solution, 0.5 mL S9 mix for metablic activation assays and 2 mL soft agar preparation were mixed and briefly incubated at 45°C (maximum 30 seconds), then plated and incubated at 37°C for 48 hours (as indicated in Ames *et al.*, 1973a, 1975; and Maron and

Ames, 1983). For Experiment 2 (pre-incubation test), test material/bacterial culture mixtures were pre-incubated in water bath at 37°C for 20 minutes, then mixed with 2 mL soft agar and plated.

Test material toxicity was assessed by visual appraisal of the condition of the bacterial background lawn; observation of a marked dose-dependent reduction in mutant counts per plate (compared with controls), and by a titer measurement. Revertant colonies for a given tester strain and activation condition were counted by automated colony counter.

3. Statistics

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated. Reporting of statistical evaluation was not provided.

4. Evaluation criteria

For acceptance of the assay, the following criteria were required to be met:

a. Negative controls within expected range, as indicated in published data (Maron and Ames, 1983) and/or internal laboratory historical control data
b. Positive controls had to show sufficient demonstration of mutagenic effect, as defined by internal laboratory historical experience/data
c. Titer determinations demonstrated sufficient bacterial density in suspensions

Acceptance of the assay was also considered appropriate where mutagenic activity was demonstrated, even in the absence of the fulfilment of criteria (b) and (c) above.

A result was considered positive where a reproducible and dose-related increase in mutant counts for at least one strain was identified (defined as approximately ≥ 2-fold increase in mutant counts compared with negative controls for TA1535, TA100 and TA98; a ≥ 3-fold increase for TA1537, and an increase of ≥100 mutant colonies for TA102. All other results were considered negative, though assessment criteria were subject to scientific judgement.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Dimethyl sulfoxide (DMSO) was selected as the solvent of choice based on the bacteriotoxic effect profile in pre-incubation experiments (not detailed in this study). In stability analyses, BYI02960 was present at acceptable concentrations in DMSO solvent at 0.01–300 mg/mL for at least 24 hours (100–104% of nominal concentrations).

B. MUTATION ASSAYS

A weak bacteriotoxic effect was noted at 5000 µg test material/plate in both experiments ±S9 activation, demonstrated by a decrease in mean colony numbers/plate when compared with negative vehicle controls and lower dose levels (Tables 1–2). No evidence of a dose-related and biologically relevant increase in revertant mutant values were observed in strains tested when compared with negative vehicle controls, with or without S9 activation.

Controls: The positive and vehicle controls fulfilled the requirements for a valid test.

Table 1
Summary of average revertants/plate after treatment with BYI02960 without activation

Compound	Conc. µg/plate	TA98		TA100		TA102		TA1535		TA1537	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
BYI02960	0	25 ± 3	17 ± 6	124 ± 26	128 ± 9	212 ± 15	220 ± 8	8 ± 1	8 ± 1	7 ± 1	7 ± 2
	16	26 ± 3	19 ± 3	128 ± 6	119 ± 7	216 12	197 ± 38	8 ± 2	9 ± 2	6 ± 1	7 ± 1
	50	27 ± 3	17 ± 6	121 ± 13	112 ± 8	218 ± 24	215 ± 20	7 ± 1	8 ± 3	6 ± 1	6 ± 2
	158	24 ± 4	19 ± 3	145 ± 29	126 ± 14	207 ± 33	227 ± 4	7 ± 1	8 ± 1	5 ± 2	7 ± 2
	500	24 ± 3	19 ± 5	135 ± 8	131 ± 13	212 ± 5	199 ± 1	7 ± 2	7 ± 1	6 ± 1	7 ± 1
	1581	28 ± 4	18 ± 4	131 ± 6	116 ± 15	220 ± 10	197 ± 6	8 ± 1	9 ± 3	5 ± 1	6 ± 1
	5000	24 ± 6	18 ± 4	116 ± 7	107 ± 5	223 ± 10	173 ± 34	7 ± 1	8 ± 3	9 ± 2	7 ± 2
NAAZ	10 20	--	--	--	--	--	--	805 ± 17 835 ± 18	629 ± 33 746 ± 20	--	--
NF	0.2 0.4	--	--	341 ± 36 406 ± 26	412 ± 9 638 ± 15	--	--	--	--	--	--
4-NDPA	0.5/10 1.0/20	68 ± 4 117 ± 14	73 ± 16 154 ± 23	--	--	--	--	--	--	35 ± 3 52 ± 2	32 ± 2 71 ± 5
MMC	0.2 0.4	--	--	--	--	657 ± 39 742 ± 31	--	--	--	--	--
Cumene	50 75	--	--	--	--	--	418 ± 15 448 ± 26	--	--	--	--

NF = Nitrofuratoin; NAAZ = Sodium azide; 4-NDPA = 4-nitro-1,2-phenylene diamine; MMC = mitomycin C; Cumene = Cumene hydroperoxide.

Data were derived from Tables 1–10, pp 58–67.

Table 2
Summary of average revertants/plate after treatment with BYI02960 with activation

Compound	Conc. µg/plate	TA98		TA100		TA102		TA1535		TA1537	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
BYI02960	0	31 ± 8	35 ± 9	198 ± 11	176 ± 6	294 ± 8	263 ± 14	12 ± 3	10 ± 1	10 ± 1	10 ± 2
	16	38 ± 3	31 ± 3	182 ± 36	164 ± 22	297 ± 12	224 ± 33	13 ± 4	11 ± 2	8 ± 2	8 ± 3
	50	25 ± 5	33 ± 4	190 ± 8	150 ± 7	314 ± 18	248 ± 55	10 ± 2	9 ± 2	10 ± 1	10 ± 1
	158	35 ± 10	32 ± 10	177 ± 10	190 ± 26	335 ± 18	268 ± 25	10 ± 3	9 ± 3	8 ± 3	7 ± 2
	500	29 ± 7	33 ± 5	213 ± 6	183 ± 15	314 ± 37	311 ± 25	11 ± 3	10 ± 1	8 ± 1	9 ± 3
	1581	26 ± 3	22 ± 4	202 ± 20	165 ± 10	288 ± 20	245 ± 5	8 ± 1	9 ± 1	7 ± 2	8 ± 2
	5000	35 ± 1	28 ± 3	188 ± 19	115 ± 27	214 ± 25	244 ± 35	7 ± 2	8 ± 3	7 ± 1	9 ± 1
2AA	3 6	2031 ± 129 2221 ± 287	917 ± 26 1065 ± 202	1719 ± 91 1881 ± 107	1405 ± 22 1914 ± 163	613 ± 35 1183 ± 207	553 ± 8 1191 ± 107	90 ± 21 59 ± 4	82 ± 8 60 ± 6	304 ± 33 135 ± 8	309 ± 29 239 ± 46

2AA= 2-aminoanthracene

Data were derived from Tables 1–10, pp 58–67.

Primary review: Australia

Secondary review: US EPA & PMRA

B.6.4.1/02	***In vitro* genotoxicity—Bacterial Assay for Gene Mutation**
Report:	Sokolowski, A. (2011) *Salmonella Typhimurium* reverse mutation assay with BYI 02960. Harlan Cytotest Cell Research GmbH, Rossdorf, Germany**.** Activity No.: TXRVP095. Harlan CCR Study ID: 1425802. Ref: M-420539-02-2. MRID 48844125. Unpubllished
Dates of work:	8–23 August 2011 (1st Amendment 17 October 2011).
Guidelines:	OPPTS 870.5100 (1998), EC No. 440/2008 B13/14 (2008), OECD No. 471 (1997).
GLP/QA:	Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary:

In independent trials of a reverse gene mutation assay, BYI 02960 (97.2% Lot No. PFV107N005) was evaluated for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 with and without an exogenous metabolic activation system (phenobarbital/β-naphthoflavone-induced rat liver S9) in two phases using the plate incorporation method (Experiment 1) and pre-incubation method (Experiment 2). The dose levels tested were 0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate for Experiment1, and 0, 33, 100, 333, 1000, 2500 and 5000 µg/plate for Experiment 2. The test substance was administered to the test system as a solution in dimethyl sulfoxide (DMSO).

A slight bacteriotoxic effect was observed at the limit dose of 5000 µg/plate in TA1537 plates with and without S9 metabolic activation in Experiment 1. The positive and vehicle controls fulfilled the requirements for a valid test. In the mutagenicity assay using both test methods, no positive mutagenic response occurred.

Under the conditions of this study, BYI 02960 was tested up to the limit dose (5000 µg/plate) and was negative for mutagenic activity in non-activated and S9-activated test systems.

The study is classified as **reliable (acceptable/guideline)** and satisfies the guideline requirements (OPPTS 870.5100; OECD 471) for *in vitro* mutagenicity (bacterial reverse gene mutation) data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960 technical
   Lot/Batch #: PFV107N005
   Purity: 97.2% (analysed 24 May 2011)
   Description: Beige powder
   CAS # 951659-40-8
   Stability of test compound: Stated to be stable for 18 months when stored at 20±10°C
2. Control materials
   Solvent control: Dimethyl sulfoxide (DMSO) for all compounds (except water for sodium azide and methyl methane sulfonate)

Positive control, non-activation:

Positive control	Dose (µg or µL/plate)	Strain(s)
Sodium azide	10	TA100 and TA1535
4-nitro-*o*-phenylene diamine	10 50	TA98 TA1537
Methyl methane sulfonate	3	TA102

Positive control, activation:

Positive control	Dose (µg/plate)	Strain
2-Aminoanthracene	2.5 10	TA98, TA100, TA1535, TA1537 TA102

3. Activation: Rat liver S9 from Male Sprague-Dawley rats (Phenobarbital/β-naphthoflavone-induced)
   Source: In-house production (Harlan CCR)
   Protein content: 33.9 mg/mL (Exp. 1 batch)
   25.7 mg/mL (Exp. 2 batch)
   Characterisation: The metabolic activation ability of the S9 was assayed for its ability to metabolize reference mutagens (2-aminoanthracene and benzo[a]pyrene).

S9 mix composition	
Phosphate buffer (pH 7.4):	100 mM
Glucose-6-phosphate:	5 mM
NADP:	4 mM
KCl:	33 mM
$MgCl_{2:}$	8 mM
S9:	10% (v/v)

4. Test organisms

Salmonella typhimurium strains TA98, TA100, TA102, TA1535, and TA1537 (Trinova Biochem GmbH, Gieβen, Germany) were properly maintained and were checked for appropriate genetic markers and sensitivity (membrane permeability, ampicillin and tetracycline resistance, spontaneous mutation).

5. Test concentrations for mutagenicity assay

Exp. 1: Plate incorporation	Concentrations of 0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 μg BYI02960/plate were evaluated in triplicate in the presence and absence of S9 activation.
Exp. 2: Pre-incubation	Concentrations of 0, 33, 100, 333, 1000, 2500 and 5000μg BYI02960/plate were evaluated in triplicate in the presence and absence of S9 activation.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

8–23 August 2011

2. Main assay methods

This study consisted of 2 independent trials, Experiment 1 (Plate incorporation, as a pre-experiment for toxicity) and Experiment 2 (pre-incubation study, confirmatory test).

In both experiments, negative vehicle control, positive controls and at least six dose levels of the test substance were plated, with overnight cultures of TA98, TA100, TA102, TA1535 and TA1537 on selective minimal agar in the presence and absence of phenobarbital/β-naphthoflavone-induced -induced rat liver S9 to evaluate the mutagenic potential of the test substance. All dose levels of test substance, negative vehicle control and positive controls were plated in triplicate. Plates were incubated for at least 48 hours at 37°C. No untreated control was used in this study.

For Experiment 1 (the plate incorporation test), 0.1 mL of test material, 0.1 mL bacterial solution, 0.5 mL S9 mix and 2 mL soft agar preparation were mixed, then plated and incubated at 37°C for at least 48 hours. For Experiment 2 (pre-incubation test), test

material/bacterial culture mixtures were pre-incubated in water bath at 37°C for 60 minutes, then mixed with 2 mL soft agar and plated for incubation at 37°C for at least 48 hours.

Revertant colonies for a given tester strain and activation condition were counted by automated colony counter, with supplementary counting by manual inspection.

3. Statistics

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated. Reporting of statistical evaluation was not provided. .

4. Evaluation criteria

For acceptance of the assay, the following criteria were required to be met:

- Regular background growth in negative and solvent controls
- Spontaneous reversion rates for negative/solvent controls within historical control data
- Positive control data produced a significant increase in mutant colony frequencies

A result was considered positive where a biologically relevant increase in mutant counts for at least one strain was identified (defined as ≥ 2-fold increase in mutant counts compared with negative controls for TA102, TA100 and TA98; a ≥ 3-fold increase for TA1535 and TA1537). For the purposes of this study, biologically relevant was defined as a dose-dependent increase at more than one concentration for a strain, or where a positive result at one concentration was reproducible in a second experiment. Additionally, a result could also be considered indicative of mutagenic potential where a dose-dependent increase in revertant colonies below the biologically relevant threshold was noted in both experiments; however, where reported values were within historical negative/solvent controls, the observed increases would not be considered evidence for mutagenicity.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Dimethyl sulfoxide (DMSO) was selected as the solvent of choice based on the known bacteriotoxic effect and test material solubility profiles. No specific stability data for the test material in solvent was provided, however, no precipitation of the test material was observed when used in the experiment.

B. MUTATION ASSAYS

In Experiment 1 (plate incorporation), a reduction in the number of TA1537 revertants (reported as a decrease below the indication factor of 0.5) was noted at 5000 µg/plate test material with and without S9 metabolic activation. No other bacteriotoxic effects were observed at other dose levels. No evidence of a dose-dependent and/or biologically relevant

mutagenic response was observed at up to and including the limit dose of 5000 μg/plate test material S9 metabolic activation (Table 1).

In Experiment 2 (pre-incubation), no evidence of bacteriotoxicity or mutagenicity was observed at up to and including the limit dose of 5000 μg/plate with or without S9 metabolic activation (Table 2).

Controls: Concurrent negative and positive controls elicited the expected responses and fulfilled the requirements for a valid test.

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable (acceptable/Guideline)

This study is compliant with OECD 471(1997) and other internationally-recognised equivalent test guidelines.

C. CONCLUSIONS: Under the conditions of this study, BYI02960 was negative for mutagenic activity up to the limit dose of 5000 μg/plate in all strains in both trials in the presence and absence of S9-activation in the *in vitro* bacterial gene mutation assay. There were no treatment-related increases in the mean number of revertants/plate in any strain (±S9). The positive controls induced marked increases in revertant colonies compared to controls in all strains in the presence and absence of S9 activation.

Accordingly, BYI02960 is negative in this test system for mutagenicity.

Table 1
Summary of average revertants/plate after treatment with BYI02960 without activation

Compound	Conc. µg or µL/plate	TA98		TA100		TA102		TA1535		TA1537	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
BYI02960	0 (DMSO)	29 ± 8	29 ± 6	139 ± 9	120 ± 13	491 ± 21	383 ± 25	16 ± 4	12 ± 1	13 ± 1	18 ± 5
	Untreated	48 ± 10	47 ± 9	173 ± 9	136 ± 19	485 ± 26	338 ± 24	20 ± 9	12 ± 4	17 ± 5	16 ± 7
	3	30 ± 2	--	136 ± 13	--	493 ± 19	--	13 ± 2	--	12 ± 3	--
	10	30 ± 8	--	141 ± 15	--	488 ± 2	--	17 ± 5	--	14 ± 2	--
	33	31 ± 6	29 ± 1	134 ± 9	132 ± 35	434 ± 30	354 ± 34	18 ± 4	13 ± 1	11 ± 1	15 ± 1
	100	32 ± 5	30 ± 6	136 ± 8	106 ± 8	452 ± 8	373 ± 14	16 ± 3	12 ± 3	12 ± 1	19 ± 4
	333	32 ± 5	27 ± 5	156 ± 16	138 ± 22	462 ± 21	380 ± 33	16 ± 4	13 ± 7	12 ± 2	16 ± 4
	1000	28 ± 2	24 ± 3	140 ± 13	96 ± 7	455 ± 24	330 ± 13	18 ± 2	15 ± 1	8 ± 2	18 ± 7
	2500	31 ± 9	31 ± 5	149 ± 8	96 ± 8	450 ± 27	321 ± 18	13 ± 3	14 ± 3	11 ± 2	17 ± 4
	5000	35 ± 6	26 ± 3	132 ± 23	89 ± 9	390 ± 25	283 ± 23	14 ± 4	15 ± 1	4 ± 1	19 ± 6
NAAZ	10	--	--	2135 ± 21	1793 ± 39	--	--	2100 ± 51	1837 ± 89	--	--
4-NOPD	10/50	275 ± 32	393 ± 26	--	--	--	--	--	--	63 ± 6	93 ± 7
MMS	3	--	--	--	--	4489 ± 32	1084 ± 27	--	--	--	--

NAAZ = Sodium azide; 4-NOPD = 4-nitro-*o*-phenylene diamine; MMS = methyl methane sulfonate.
Data were derived from Tables 14.1–14.2, pp 23–24.

Table 2 Summary of average revertants/plate after treatment with BYI02960 with activation

Compound	Conc. µg or µL/plate	TA98		TA100		TA102		TA1535		TA1537	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
BYI02960	0 (DMSO)	39 ± 9	40 ± 11	174 ± 16	122 ± 7	633 ± 27	366 ± 24	20 ± 2	20 ± 2	13 ± 1	20 ± 1
	Untreated	37 ± 7	42 ± 5	162 ± 38	157 ± 10	540 ± 78	356 ± 23	17 ± 3	15 ± 5	24 ± 2	18 ± 5
	3	40 ± 9	--	175 ± 6	--	610 ± 56	--	19 ± 6	--	16 ± 5	--
	10	36 ± 8	--	152 ± 6	--	611 ± 26	--	19 ± 6	--	13 ± 3	--
	33	40 ± 6	42 ± 6	159 ± 24	115 ± 10	612 ± 43	433 ± 20	21 ± 1	24 ± 7	14 ± 1	18 ± 2
	100	39 ± 3	37 ± 6	163 ± 1	122 ± 14	649 ± 34	385 ± 15	21 ± 7	20 ± 8	15 ± 1	20 ± 4
	333	38 ± 6	47 4	164 ± 21	122 ± 4	622 ± 59	449 ± 50	20 ± 1	20 ± 7	15 ± 6	22 ± 2
	1000	39 ± 7	44 ± 7	172 ± 12	109 ± 6	629 ± 25	487 ± 55	20 ± 3	20 ± 3	15 ± 3	17 ± 5
	2500	44 ± 4	34 ± 4	153 ± 6	118 ± 5	636 ± 68	411 ± 40	19 ± 3	18 ± 6	14 ± 2	15 ± 4
	5000	36 ± 10	37 ± 8	160 ± 16	106 ± 13	543 ± 79	278 ± 2	18 ± 2	17 ± 4	5 ± 2	19 ± 4
2-AA	2.5/10	2932 ± 127	1612 ± 26	2911 ± 47	2218 ± 182	2782 ± 74	1897 ± 10	407 ± 78	264 ± 21	344 ± 24	246 ± 8

2-AA = 2-aminoanthracene
Data were derived from Tables 14.1–14.2, pp 23–24.

Primary review: Australia
Secondary review: US EPA & PMRA

B.6.8.3/10 *In vitro* genotoxicity—Bacterial Assay for Gene Mutation

Report:	Sokolowski, A (2010a). *Salmonella Typhimurium* reverse mutation assay with BCS-AA56716 (Metabolite of BYI 02960). Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. Harlan CCR Study ID: 1351101. Ref: M-409724-01-2. MRID 48844126. Unpublished.
Dates of work:	16 August – 3 September 2010; Final report 30 September 2010.
Guidelines:	OPPTS 870.5100 (1998), EC No. 440/2008 B13/14 (2008), OECD No. 471 (1997).
GLP/QA:	Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary:

In independent trials of a reverse gene mutation assay, BCS-AA56716 (difluoroacetic acid) (99.6%, Lot No. BCOO 5984-4-11) was evaluated for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 with and without an exogenous metabolic activation system (phenobarbital/β-naphthoflavone-induced rat liver S9) in two phases using the plate incorporation method (Experiment 1) and pre-incubation method (Experiment 2). The dose levels tested were 0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate for Experiment1, and 0, 33, 100, 333, 1000, 2500 and 5000 µg/plate for Experiment 2. The test substance was administered to the test system as a solution in dimethyl sulfoxide (DMSO).

No precipitation of the test material was observed up to and including the limit dose of 5000 µg/plate in both experiments. The positive and vehicle controls fulfilled the requirements for a valid test. In the mutagenicity assay using both test methods, no positive mutagenic response occurred.

Under the conditions of this study, BCS-AA56716 was tested up to the limit dose and was negative for mutagenic activity in non-activated and S9-activated test systems.

The study is classified as **reliable (acceptable/guideline)** and satisfies the guideline requirements (OECD 471 and equivalents) for *in vitro* mutagenicity (bacterial reverse gene mutation) data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BCS-AA56716 (metabolite of BYI 02960)
   Lot/Batch #: BCOO 5984-4-11
   Purity: 99.6%
   Description: Colourless liquid
   CAS # 21543-49-7
   Stability of test compound: CoA stated that the test material was stable for 6 months when stored at 25±5°C. The stability in solvents was not discussed in the report.
2. Control materials
   Solvent control: Dimethyl sulfoxide (DMSO) at 100 µL/plate for all materials

Positive control, non-activation:

Positive control	Dose (µg or µL/plate)	Strain(s)
Sodium azide	10	TA100 and TA1535
4-nitro-*o*-phenylene diamine	10 50	TA98 TA1537
Methyl methane sulfonate	3	TA102

Positive control, activation:

Positive control	Dose (µg/plate)	Strain
2-Aminoanthracene	2.5 10	TA98, TA100, TA1535, TA1537 TA102

3. Activation: Rat liver S9 from Male Sprague-Dawley rats (Phenobarbital/β-naphthoflavone-induced)
   Source: In-house production (Harlan CCR)
   Protein content: 34.7 mg/mL (Exp. 1 batch)
   35.4 mg/mL (Exp. 2 batch)
   Characterisation: The metabolic activation ability of the S9 was assayed for its ability to metabolize reference mutagens (2-aminoanthracene and benzo[a]pyrene).

S9 mix composition	
Phosphate buffer (pH 7.4):	100 mM
Glucose-6-phosphate:	5 mM
NADP:	4 mM
KCl:	33 mM
$MgCl_2$:	8 mM
S9:	10% (v/v)

4. Test organisms

Salmonella typhimurium strains TA98, TA100, TA102, TA1535, and TA1537 (Trinova Biochem GmbH, Gieβen, Germany) were properly maintained and were checked for appropriate genetic markers and sensitivity (membrane permeability, ampicillin and tetracycline resistance, spontaneous mutation).

5. Test concentrations for mutagenicity assay

Exp. 1: Plate incorporation	Concentrations of 0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 μg BCS-AA56716 /plate were evaluated in triplicate in the presence and absence of S9 activation.
Exp. 2: Pre-incubation	Concentrations of 0, 33, 100, 333, 1000, 2500 and 5000μg BCS-AA56716 /plate were evaluated in triplicate in the presence and absence of S9 activation.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

16 August – 3 September 2010

2. Main assay methods

This study consisted of 2 independent trials, Experiment 1 (Plate incorporation, as a pre-experiment for toxicity) and Experiment 2 (pre-incubation study, confirmatory test).

In both experiments, negative vehicle control, positive controls and at least six dose levels of the test substance were plated, with overnight cultures of TA98, TA100, TA102, TA1535 and TA1537 on selective minimal agar in the presence and absence of phenobarbital/β-naphthoflavone-induced -induced rat liver S9 to evaluate the mutagenic potential of the test substance. All dose levels of test substance, negative vehicle control and positive controls were plated in triplicate. Plates were incubated for at least 48 hours at 37°C. No untreated control was used in this study.

For Experiment 1 (the plate incorporation test), 0.1 mL of test material, 0.1 mL bacterial solution, 0.5 mL S9 mix (or phosphate buffer) and 2 mL soft agar preparation were mixed, then plated and incubated at 37°C for at least 48 hours. For Experiment 2 (pre-

incubation test), test material/bacterial culture mixtures and buffer/S9 mix were pre-incubated in water bath at 37°C for 60 minutes, then mixed with 2 mL soft agar and plated for incubation at 37°C for at least 48 hours.

Revertant colonies for a given tester strain and activation condition were counted by automated colony counter, with supplementary counting by manual inspection.

3. Statistics

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated. Reporting of statistical evaluation was not provided.

4. Evaluation criteria

For acceptance of the assay, the following criteria were required to be met:

- Regular background growth in negative and solvent controls
- Spontaneous reversion rates for negative/solvent controls within historical control data
- Positive control data produced a significant increase in mutant colony frequencies

A result was considered positive where a biologically relevant increase in mutant counts for at least one strain was identified (defined as ≥ 2-fold increase in mutant counts compared with negative controls for TA102, TA100 and TA98; a ≥ 3-fold increase for TA1535 and TA1537). For the purposes of this study, biologically relevant was defined as a dose-dependent increase at more than one concentration for a strain, or where a positive result at one concentration was reproducible in a second experiment. Additionally, a result could also be considered indicative of mutagenic potential where a dose-dependent increase in revertant colonies below the biologically relevant threshold was noted in both experiments; however, where reported values were within historical negative/solvent controls, the observed increases would not be considered evidence for mutagenicity.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Dimethyl sulfoxide (DMSO) was selected as the solvent of choice based on solubility and bacterial cytotoxicity data. No stability/solubility data for the test material in solvent were presented in the study

B. MUTATION ASSAYS

In both experiments, no evidence of mutagenicity was observed (i.e. no increased frequency of revertant colonies with test material treatment) with or without S9 activation (Tables 1–2).

In both experiments, no growth inhibition of *Salmonella* bacterial strains and test material precipitation was observed with or without S9 activation.

Controls: The positive and vehicle controls fulfilled the requirements for a valid test.

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 471(1997) and other internationally-recognised equivalent test guidelines.

C. CONCLUSIONS: Under the conditions of this study, BCS-AA56716 was negative for mutagenic activity up to the limit dose of 5000 μg/plate in all strains in both trials in the presence and absence of S9-activation in the *in vitro* bacterial gene mutation assay. There were no treatment-related increases in the mean number of revertants/plate in any strain (±S9). The positive controls induced marked increases in revertant colonies compared to controls in all strains in the presence and absence of S9 activation.

Accordingly, BCS-AA56716 is negative in this test system for mutagenicity.

Table 1
Summary of average revertants/plate after treatment with BCS-AA56716 without activation

Compound	Conc. µg or µL/plate	TA98		TA100		TA102		TA1535		TA1537	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
BCS-AA56716	Untreated	29 ± 6	31 ± 5	125 ± 17	147 ± 19	421 ± 29	401 ± 5	17 ±3	18 ± 10	11 ± 4	9 ± 6
	0	25 ± 4	38 ± 14	120 ± 14	114 ± 5	388 ± 12	393 ± 47	13 ± 3	15 6	11 ± 5	12 ± 4
	3	27 ± 6	--	121 ± 18	--	371 ± 21	--	15 ± 2	--	10 ± 2	--
	10	28 ± 5	--	128 ± 19	--	433 ± 24	--	13 ± 3	--	13 ± 2	--
	33	28 ± 7	31 ± 3	115 ± 3	122 ± 6	421 ± 18	396 ± 17	14 ± 3	17 ± 4	12 ± 5	10 ± 2
	100	29 ± 2	33 ± 9	129 ± 7	106 ± 7	411 ± 14	415 ± 22	13 ± 2	15 ± 4	13 ± 4	15 ± 3
	333	32 ± 3	25 ± 4	116 ± 2	126 ± 9	379 ± 8	371 ± 18	14 ± 2	19 ± 2	9 ± 1	12 ± 4
	1000	34 ± 4	36 ± 2	115 ± 18	116 ± 13	408 ± 15	416 ± 24	13 ± 2	11 ± 3	12 ± 5	7 ± 2
	2500	36 ± 3	35 ± 4	111 ± 4	115 ± 12	427 ± 25	429 ± 17	15 ± 0	14 ± 3	10 ± 5	13 ± 3
	5000	25 ± 2	30 ± 2	123 ± 4	118 ± 13	418 ± 13	367 ± 16	18 ± 4	16 ± 3	7 ± 3	13 ± 4
NAAZ	10	--	--	2188 ± 169	1684 ± 179	--	--	1870 ± 58	1631 ± 25	--	--
4-NOPD	10/50	320 ± 20	408 ± 14	--	--	--	--	--	--	77 ± 6	79 ± 8
MMS	3	--	--	--	--	3613 ± 251	1956 ± 25	--	--	--	--

NAAZ = Sodium azide; 4-NOPD = 4-nitro-*o*-phenylene diamine; MMS = methyl methane sulfonate.

Data were derived from Tables 14.1–14.2, pp 22–23.

Table 2
Summary of average revertants/plate after treatment with BCS-AA56716 with activation

Compound	Conc. µg or µL/plate	TA98		TA100		TA102		TA1535		TA1537	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
BCS-AA56716	Untreated	41 ± 9	41 ± 9	155 ± 32	173 ± 13	569 ± 18	565 ± 23	24 ± 2	24 ± 3	13 ± 5	15 ± 4
	0	42 ± 1	37 ± 3	138 ± 13	138 ± 3	587 ± 31	484 ± 38	18 ± 5	17 ± 4	18 ± 8	12 ± 4
	3	42 ± 8	--	153 ± 16	--	595 ± 41	--	17 ± 4	--	19 ± 3	--
	10	36 ± 5	--	140 ± 7	--	608 ± 51	--	17 ± 5	--	16 ± 6	--
	33	49 ± 2	36 ± 10	144 ± 6	145 ± 18	641 ± 19	562 ± 24	16 ± 1	19 ± 2	18 ± 4	14 ± 5
	100	41 ± 4	36 ± 11	143 ± 6	138 ± 23	613 ± 28	574 ± 27	20 ± 4	20 ± 7	15 ± 2	11 ± 4
	333	47 ± 2	37 ± 3	126 ± 4	133 ± 8	562 ± 36	510 ± 59	20 ± 3	14 ± 2	15 ± 5	15 ± 2
	1000	38 ± 3	40 ± 5	142 ± 8	126 ± 4	592 ± 35	521 ± 11	23 ± 5	25 ± 8	15 ± 4	12 ± 4
	2500	45 ± 2	40 ± 3	152 ± 20	135 ± 17	620 ± 22	531 ± 35	20 ± 1	18 ± 1	16 ± 2	18 ± 4
	5000	48 ± 6	41 ± 8	147 ± 10	141 ± 11	578 ± 38	482 ± 34	20 ± 2	28 ± 8	15 ± 1	15 ± 6
2-AA	2.5/10	2861 ± 181	1730 ± 352	3435 ± 175	2007 ± 84	2457 ± 282	2739 ± 175	437 ± 23	367 ± 7	429 ± 40	230 ± 22

2-AA = 2-aminoanthracene

Data were derived from Tables 14.1–14.2, pp 22–23.

Primary review: Australia
Secondary review: US EPA & PMRA

B.6.8.3/11 *In vitro* genotoxicity—Bacterial Assay for Gene Mutation

Report:	Sokolowski, A (2010b). *Salmonella Typhimurium* reverse mutation assay with BYI 02960-difluoroethyl-amino-furanone (Metabolite of BYI 02960). Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. Harlan CCR Study ID: 1399701. Ref: M-409728-01-2. MRID 48844127. Unpublished .
Dates of work:	31 March – 14 April 2011; Final report 24 May 2011.
Guidelines:	OPPTS 870.5100 (1998), EC No. 440/2008 B13/14 (2008), OECD No. 471 (1997).
GLP/QA:	Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary:

In independent trials of a reverse gene mutation assay, BYI 02960-difluoroethyl-amino-furanone (BCS-CC98193) (99.6%, Lot No. BCOO 5984-4-11) was evaluated for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 with and without an exogenous metabolic activation system (phenobarbital/β-naphthoflavone-induced rat liver S9) in two phases using the plate incorporation method (Experiment 1) and pre-incubation method (Experiment 2). The dose levels tested were 0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate for Experiment1, and 0, 33, 100, 333, 1000, 2500 and 5000 µg/plate for Experiment 2. The test substance was administered to the test system as a solution in dimethyl sulfoxide (DMSO).

No precipitation of the test material was observed up to and including the limit dose of 5000 µg/plate in both experiments. The positive and vehicle controls fulfilled the requirements for a valid test, apart from a minor decrease in the expected revertant colony count with the untreated control for bacterial strain TA102 without activation which was not considered biologically relevant in the context of this study and did not affect the validity of study results. In the mutagenicity assay using both test methods, no positive mutagenic response occurred with BYI 02960-difluoroethyl-amino-furanone.

Under the conditions of this study, BYI 02960-difluoroethyl-amino-furanone was tested up to the limit dose and was negative for mutagenic activity in non-activated and S9-activated test systems.

The study is classified as **reliable (acceptable/guideline)** and satisfies the guideline requirements (OECD 471 and equivalents) for *in vitro* mutagenicity (bacterial reverse gene mutation) data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BCS-CC98193 (BYI-20960-difuoroethyl-amino-furanone: metabolite of BYI 02960)
   Lot/Batch #: NLL 8671-6-1
   Purity: 98.5%
   Description: White Powder
   CAS # 21543-49-7
   Stability of test compound: CoA stated that the test material was stable for 6 months when stored at 20±10°C. The stability in solvents was not discussed in the report.
2. Control materials
   Solvent control: Dimethyl sulfoxide (DMSO) at 100 µL/plate for all materials

Positive control, non-activation:

Positive control	Dose (µg or µL/plate)	Strain(s)
Sodium azide	10	TA100 and TA1535
4-nitro-*o*-phenylene diamine	10 50	TA98 TA1537
Methyl methane sulfonate	3	TA102

Positive control, activation:

Positive control	Dose (µg/plate)	Strain
2-Aminoanthracene	2.5 10	TA98, TA100, TA1535, TA1537 TA102

3. Activation: Rat liver S9 from Male Sprague-Dawley rats (Phenobarbital/β-naphthoflavone-induced)
   Source: In-house production (Harlan CCR)
   Protein content: 30.7 mg/mL
   Characterisation: The metabolic activation ability of the S9 was assayed for its ability to metabolize reference mutagens (2-aminoanthracene and benzo[a]pyrene).

S9 mix composition	
Phosphate buffer (pH 7.4):	100 mM
Glucose-6-phosphate:	5 mM
NADP:	4 mM
KCl:	33 mM
$MgCl_2$:	8 mM
S9:	10% (v/v)

4. Test organisms

Salmonella typhimurium strains TA98, TA100, TA102, TA1535, and TA1537 (Trinova Biochem GmbH, Gieβen, Germany) were properly maintained and were checked for appropriate genetic markers and sensitivity (membrane permeability, ampicillin and tetracycline resistance, spontaneous mutation).

5. Test concentrations for mutagenicity assay

Exp. 1: Plate incorporation	Concentrations of 0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 μg BYI-20960-difuoroethyl-amino-furanone /plate were evaluated in triplicate in the presence and absence of S9 activation.
Exp. 2: Pre-incubation	Concentrations of 0, 33, 100, 333, 1000, 2500 and 5000μg BYI-20960-difuoroethyl-amino-furanone /plate were evaluated in triplicate in the presence and absence of S9 activation.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

31 March – 14 April 2011

2. Main assay methods

This study consisted of 2 independent trials, Experiment 1 (Plate incorporation, as a pre-experiment for toxicity) and Experiment 2 (pre-incubation study, confirmatory test).

In both experiments, negative vehicle control, positive controls and at least six dose levels of the test substance were plated, with overnight cultures of TA98, TA100, TA102, TA1535 and TA1537 on selective minimal agar in the presence and absence of phenobarbital/β-naphthoflavone-induced rat liver S9 to evaluate the mutagenic potential of the test substance. All dose levels of test substance, negative vehicle control and positive controls were plated in triplicate. Plates were incubated for at least 48 hours at 37°C. No untreated control was used in this study.

For Experiment 1 (the plate incorporation test), 0.1 mL of test material, 0.1 mL bacterial solution, 0.5 mL S9 mix (or phosphate buffer) and 2 mL soft agar preparation were mixed, then plated and incubated at 37°C for at least 48 hours. For Experiment 2 (pre-

incubation test), test material/bacterial culture mixtures and buffer/S9 mix were pre-incubated in water bath at 37°C for 60 minutes, then mixed with 2 mL soft agar and plated for incubation at 37°C for at least 48 hours.

Revertant colonies for a given tester strain and activation condition were counted by automated colony counter, with supplementary counting by manual inspection.

3. Statistics

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated. Reporting of statistical evaluation was not provided.

4. Evaluation criteria

For acceptance of the assay, the following criteria were required to be met:

- Regular background growth in negative and solvent controls
- Spontaneous reversion rates for negative/solvent controls within historical control data
- Positive control data produced a significant increase in mutant colony frequencies

A result was considered positive where a biologically relevant increase in mutant counts for at least one strain was identified (defined as ≥ 2-fold increase in mutant counts compared with negative controls for TA102, TA100 and TA98; a ≥ 3-fold increase for TA1535 and TA1537). For the purposes of this study, biologically relevant was defined as a dose-dependent increase at more than one concentration for a strain, or where a positive result at one concentration was reproducible in a second experiment. Additionally, a result could also be considered indicative of mutagenic potential where a dose-dependent increase in revertant colonies below the biologically relevant threshold was noted in both experiments; however, where reported values were within historical negative/solvent controls, the observed increases would not be considered evidence for mutagenicity.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Dimethyl sulfoxide (DMSO) was selected as the solvent of choice based on solubility and bacterial cytotoxicity data. No stability/solubility data for the test material in solvent were presented in the study

B. MUTATION ASSAYS

In both experiments, no evidence of mutagenicity was observed (i.e. no increased frequency of revertant colonies with test material treatment) with or without S9 activation.

In both experiments, no growth inhibition of *Salmonella* bacterial strains and test material precipitation was observed with or without S9 activation (Tables 1–2) up to the limit dose.

Controls: Most of the positive and vehicle controls fulfilled the requirements for a valid test. The untreated control for TA102 with S9 activation was marginally below the laboratory historical control lower limit in Experiment 1 (mean value of 324 revertant colonies vs lower limit of 328 in historical controls). However, noting the marginal nature of this finding, and the lack of this effect in the second experiment, the study director considered that this was not biologically relevant in the context of this study. The evaluator concurs with this analysis and considers that the effect described does not affect the validity of study results.

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 471(1997) and other internationally-recognised equivalent test guidelines.

C. CONCLUSIONS: Under the conditions of this study, BYI-20960-difuoroethyl-amino-furanone was negative for mutagenic activity up to the limit dose of 5000 μg/plate in all strains in both trials in the presence and absence of S9-activation in the *in vitro* bacterial gene mutation assay. There were no treatment-related increases in the mean number of revertants/plate in any strain (±S9). The positive controls induced marked increases in revertant colonies compared to controls in all strains in the presence and absence of S9 activation.

Accordingly, BYI-20960-difuoroethyl-amino-furanone is negative in this test system for mutagenicity.

Table 1
Summary of average revertants/plate after treatment with BYI-20960-difuoroethyl-amino-furanone without activation

Compound	Conc. µg or µL/plate	TA98		TA100		TA102		TA1535		TA1537	
		Exp. 1	Exp. 2*	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
BCS-AA56716	0 (DMSO)	28 ± 5	26 ± 3	141 ± 11	156 ± 6	330 ± 5	432 ± 2	13 ± 5	16 ± 2	16 ± 2	19 ± 2
	Untreated	26 ± 4	27 ± 3	144 ± 12	186 ± 10	320 ± 12	445 ± 24	13 ± 1	13 ± 2	20 ± 1	20 ± 7
	3	30 ± 3	--	127 ± 14	--	307 ± 16	--	13 ± 4	--	15 ± 3	--
	10	31 ± 4	--	136 ± 5	--	340 ± 12	--	11 ± 3	--	16 ± 3	--
	33	30 ± 3	27 ± 4	137 ± 10	142 ± 11	360 ± 24	477 ± 18	14 ± 2	17 ± 3	19 ± 6	20 ± 4
	100	29 ± 2	23 ± 4	137 ± 8	141 ± 5	351 ± 22	459 ± 6	15 ± 4	16 ± 2	19 ± 8	22 ± 2
	333	26 ± 6	26 ± 1	124 ± 4	151 ± 5	313 ± 8	476 ± 10	13 ± 1	18 ± 4	17 ± 7	26 ± 2
	1000	29 ± 7	30 ± 2	135 ± 12	158 ± 7	328 ± 13	427 ± 19	12 ± 2	17 ± 6	16 ± 1	20 ± 4
	2500	28 ± 6	28 ± 2	131 ± 15	158 ± 10	326 ± 14	456 ± 10	14 ± 1	19 ± 4	15 ± 2	21 ± 2
	5000	27 ± 4	28 ± 2	143 ± 15	152 ± 22	320 ± 20	473 ± 24	17 ± 3	16 ± 4	13 ± 3	20 ± 1
NAAZ	10	--	--	2167 ± 251	2464 ± 83	--	--	1857 ± 114	2237 ± 96	--	--
4-NOPD	10/50	408 ± 50	382 ± 31	--	--	--	--	--	--	65 ± 4	106 ± 13
MMS	3	--	--	--	--	4464 ± 55	2091 ± 44	--	--	--	--

NAAZ = Sodium azide; 4-NOPD = 4-nitro-*o*-phenylene diamine; MMS = methyl methane sulfonate. * Manually counted due to extensive bacterial growth.

Data were derived from Tables 14.1–14.2, pp 22–23.

Table 2
Summary of average revertants/plate after treatment with BYI-20960-difuoroethyl-amino-furanone with activation

Compound	Conc. µg or µL/plate	TA98		TA100		TA102		TA1535		TA1537	
		Exp. 1	Exp. 2*	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
BCS-AA56716	0 (DMSO)	42 ± 8	40 ± 1	149 ± 7	160 ± 11	357 ± 6	632 ± 27	27 ± 1	21 ± 2	24 ± 3	20 ± 1
	Untreated	51 ± 8	38 ± 4	147 ± 6	216 ± 8	324 ± 14	650 ± 7	20 ± 4	22 ± 8	25 ± 5	26 ± 4
	3	38 ± 6	--	138 ± 9	--	371 ± 25	--	26 ± 6	--	22 ± 1	--
	10	41 ± 5	--	159 ± 10	--	390 ± 14	--	22 ± 5	--	24 ± 5	--
	33	39 ± 11	40 ± 4	153 ± 7	166 ± 9	395 ± 13	595 ± 8	26 ± 10	19 ± 2	26 ± 6	24 ± 7
	100	41 ± 7	40 ± 12	154 ± 15	173 ± 20	393 ± 30	641 ± 29	23 ± 4	20 ± 3	25 ± 4	18 ± 3
	333	45 ± 6	41 ± 5	147 ± 9	196 ± 24	377 ± 42	670 ± 29	24 ± 2	22 ± 4	27 ± 2	21 ± 7
	1000	47 ± 4	37 ± 3	153 ± 15	174 ± 10	423 ± 14	644 ± 36	25 ± 4	19 ± 2	22 ± 5	22 ± 7
	2500	34 ± 9	40 ± 4	150 ± 5	169 ± 27	380 ± 32	648 ± 38	27 ± 4	20 ± 1	24 ± 7	25 ± 7
	5000	41 ± 8	35 ± 4	160 ± 15	186 ± 27	386 ± 7	655 ± 10	29 ± 7	21 ± 4	30 ± 4	25 ± 5
2-AA	2.5/10	2409 ± 64	2532 ± 94	2583 ± 201	2402 ± 146	2553 ± 216	4314 ± 630	301 ± 20	403 ± 35	298 ± 29	302 ± 11

2-AA = 2-aminoanthracene. * Manually counted due to extensive bacterial growth

Data were derived from Tables 14.1–14.2, pp 22–23.

Primary Review: Australia
Secondary Review: US EPA & PMRA

B.6.4.2 *In vitro* genotoxicity: Test for gene mutation in mammalian cells

Report:	Entian, G. (2009); BYI 02960: V79/HPRT Test *In Vitro* for the Detection of Induced Forward Mutations. Bayer Schering Pharma AG, Wuppertal, Germany. Study ID: T 1080067. Report No.: AT05625. Ref: M-359743-01-2. MRID 48844128. Unpublished.
Dates of work:	21 April – 25 September 2009. Final Report 29 October 2009.
Guidelines:	US EPA OPPTS 870.5300 (1998); EEC 2000/32/EC, Method B17 (2000); OECD TG 476 (1998)
GLP/QA:	OECD, US EPA, EC and JMAFF GLP-compliant Signed and dated Quality Assurance and Data Confidentiality were presented in the report.

Executive summary:

In a Chinese Hamster V79 cell/HPRT mutation assay (Ref: M-359743-01-2), BYI 02960 (96.2% purity) was tested in the presence and absence of an exogenous metabolic activation system (Aroclor-induced rat liver S9) for its potential to cause gene mutation at the HPRT locus. Following a preliminary toxicity assay, duplicate flasks of exponentially growing V79 cells were exposed for 5 hours at 37 ± 1°C to the test substance at concentrations of 0, 46, 92, 184, 368, 736, 1472 and 2944 µg/mL for 5 hours (±S9) at 37°C. The highest concentration level was set based on the lack of test substance precipitation at a concentration greater than the limit dose of 10 mM. Cells were then independently subcultured for assessment of cytotoxicity (cloning efficiency) and for expression and selection of the 6-thioguanine (2-amino-6-mercaptopurine)-resistant phenotype. The test substance was dissolved in dimethyl sulfoxide (DMSO) at a maximum concentration of 300 mg/mL. Ethyl methanesulfonate (EMS) and dimethylbenzanthracene (DMBA) were used as positive controls for the non-activated and activated test systems, respectively.
In the mutagenesis assay, visible precipitate was observed at the limit dose of 2944 µg/mL. However, no excessive cytotoxicity was observed at any concentration tested, and no concentration- and treatment-related increases in mutant frequency were observed.
BYI 02960 was negative in the non-activated and S9-activated test systems in the V79/HPRT mutation assay.
This study is reliable (acceptable/guideline) and satisfies the requirements for an *in-vitro* mammalian cell gene mutation assay (OPPTS 870.5300; OECD 476).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   Lot/Batch #: 2009-000239
   Purity: 96.2%
   Description: Fine crystalline brownish powder
   CAS #: 951659-40-8
   Stability of test compound: Results from stability analyses in DMSO indicated that BYI 02960 was stable at up to 300 mg/mL for 24 hours at room temperature
2. Control materials
   Solvent control: Dimethyl sulfoxide (DMSO)
   Positive, non-activation: Ethyl methanesulfonate (EMS) at a final concentration of 900 µg/mL
   Positive, activation: Dimethylbenzanthracene (DMBA) in DMSO at a final concentration of 20 µg/mL
3. Activation: Rat liver S9 from Male Sprague-Dawley rats (Aroclor 1254-induced)
   Lot number: Not specified (date of preparation 26 January 2009)
   Source: In-house preparation
   Protein content: 24.5 mg/mL
   Characterisation: The metabolic activation ability of the S9 was characterised by its ability to metabolize 20 µg/mL DMBA.

   S9 mix composition
   NADP: 4 mM
   Glucose-6-phosphate: 5 mM
   KCl: 33 mM
   $MgCl_2$: 8 mM
   Na phosphate buffer (pH 7.4): 150 mM
   S9 homogenate: 40% (v/v)

4. Test cells

Chinese Hamster V79 cells were obtained from Dr. Utesch (Merck AG, Darmstadt) on 11 March 2008. Cell stocks were created and stored in liquid nitrogen, and cells were raised from stock and maintained in tissue culture vessels at 37°C in 5% CO_2 atmosphere, with

replating and spontaneous mutation suppression carried out as required to ensure proper cell karyotype and 6-TG sensitivity prior to study commencement.

5. Culture medium

Commercial PAA Ready Mix medium, containing Eagle's minimal essential medium and 1% each of L-glutamine, MEM-vitamins, MEM NEAA, penicillin/streptomycin mixture, supplemented with either 2% foetal calf serum (FCS) during treatment with test material, or 10 % FCS while used for cell culture maintenance and growth.

6. Locus examined

Hypoxanthine-guanine-phosphoribosyl transferase (HPRT) – the selection agent used was 6-thioguanine (6TG, 2-amino-6-mercaptopurine) at a final concentration of 10 μg/mL in culture.

7. Test compound concentrations used

Preliminary cytotoxicity assay:	Concentrations of 0, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 2000 and 3000 μg/mL test material were evaluated in the presence and absence of S9 activation. Exposure time was 5 hours (±S9) at 37°C .
Mutagenesis assay:	Concentrations of 0, 46, 92, 184, 368, 736, 1472 and 2944 μg/mL test material were evaluated in the presence and absence of S9 activation. The assay was repeated once. Exposure time was 5 hours (±S9) at 37°C.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion:

21 April – 25 September 2009

2. Preliminary cytotoxicity assay

V79 cells were exposed for 5 hours to vehicle alone and nine concentrations of test substance ranging from 15.6 to 3000 μg/mL (exceeding the limit dose of 10 mM) in both the absence and presence of S9-activation for evaluation of test substance effect on relative cloning efficiency (CE). Culture medium containing 2% FCS was used during test substance exposure, and culture medium containing 10% FCS was used at all other points during the assay.

3. Mutagenesis assay

V79 cells were exposed for 5 hours to the vehicle alone, appropriate positive controls and seven concentrations of test substance in duplicate in both the absence and presence of S9-activation at 37 ± 1°C. After 5 hours, the cells were washed with phosphate-buffered saline, trypsinised and re-plated in culture medium (10% FCS) for cytotoxicity and phenotypic expression/selection evaluation (see below).

Cytotoxicity: The replicates from each treatment condition were detached using trypsin and subcultured in triplicate at a density of 200 cells/60 mm dish. After approximately 6–8 days incubation, the colonies were fixed with methanol, stained with Giemsa, counted and cloning efficiency determined.

Phenotypic expression/selection: The cultures from each dose level were detached using trypsin and cultured at a density of 1.5×10^6 cells/flask initially, with sub-culturing at a density of 1.5×10^6 cells/flask three days after exposure to test substance. Six days after exposure to test substance (i.e. at the end of the expression period), cultures were re-seeded into plates (3×10^5 cells/100 mm dish, in quintuplicate) containing medium with 10 μM 6-thioguanine (TG, 2-amino-6-mercaptopurine) for mutant selection. Additionally, for cloning efficiency determination at the time of selection, 200 cells/60 mm dish were plated in triplicate from the same initial culture. After 6–8 days of incubation, colonies from the various plates were fixed, stained and counted for both cloning efficiency and mutant selection.

4. Statistics

Acceptable data (as defined by evaluation criteria) were initially included in a weighted analysis of variance, followed by pair-wise comparison with the negative control by the Dunnett test ($p < 0.05$).

5. Evaluation criteria

The study was considered valid and acceptable if the following criteria were met:

- The average cloning efficiency of the negative controls should be at least 50%
- The average mutant frequency of negative controls should not exceed 25×10^{-6} cells
- The mutant frequency difference between negative (solvent) controls and untreated controls should be limited (i.e. should be generally $< 5\times10^6$)
- The positive control should induce an average mutant frequency at $\geq 3\times$ the negative control
- The highest test substance concentration should induce cytotoxicity of ~80–90% (if not limited by solubility) or should be at a concentration where test substance precipitation occurred. The cell survival index of the lowest concentration of test substance used should be comparable to untreated controls.
- At least five dishes/culture should be available for calculation of mutant frequency, and the indices for relative survival to treatment, relative population growth and absolute cloning efficiency of negative controls and treated groups should be ≥10 %.

The criteria above could be over-ruled with good scientific judgement.

The study results were interpreted as follows:

- A positive result was defined as a concentration-related, reproducible (in parallel cultures) increase in mutant frequencies. The biological relevance of the finding was also considered, with an indication that the increase in mutant frequencies should be at least 2–3 times that of the concurrent negative control in the trial. If the result was repeated in a separate trial, then the test substance was considered mutagenic. A positive result would be considered valid only if cellular osmolarity in treated cells showing increased mutant frequencies was similar to negative controls.
- An equivocal result was defined as the absence of a concentration-related increase in mutant frequencies, but at least one or more concentrations produced a

reproducible and biologically relevant increase in mutant frequencies across all trials.

- A negative result was defined as the absence of a reproducible and biologically relevant increase in mutant frequency.

The criteria above could be over-ruled with good scientific judgement.

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

DMSO was determined to be the solvent of choice based on the solubility of the test substance and compatibility with the target cells. The test substance was soluble in DMSO at a maximum concentration of 300 mg/mL. Preliminary cytotoxicity testing at up to 3000 µg/mL did not reveal concentration-related changes in relative cell survival, and no precipitation of test material was observed at tested dose levels, indicating the suitability of the selected dose series for the main experiments.

B. MUTAGENESIS ASSAY

The results from the main study assay are summarized in Tables 1 and 2 below. Absolute cloning efficiency in negative controls ranged from 75.9–89.2% (–S9) and 73.1–85.9% (+S9), indicating suitable cloning conditions for the main experiments. In cultures tested at up to the limit dose of 2944 µg/mL (equivalent to 10 mM) both with and without metabolic activation, no evidence of treatment-related and concentration-related increases in mutant frequency was observed. Visible precipitate was present at the limit dose of 2944 µg/mL in the treatment medium. While some variability was observed in cell viability, survivability and growth at this limit dose level, observed effects did not affect scoring for mutant frequency.

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 476 (1997) and other internationally-recognised equivalent test guidelines.

C. CONCLUSIONS: BYI 02960 was negative for mutagenic activity up to the limit dose of 2944 µg/mL (equivalent to 10 mM) in both trials in V79 Chinese Hamster cells in the presence and absence of S9-activation in the *in vitro* mammalian cell gene mutation assay. There were no treatment-related increases in mutation frequencies. The positive controls induced marked increases in revertant colonies compared to controls in the presence and absence of S9 activation.

Accordingly, BYI 02960 is negative in this test system for mutagenicity.

Table 1: HPRT test outcomes after treatment of V79 cells with BYI 02960 (–S9 metabolic activation)

Dose level (µg/mL)	Relative survival to treatment (% of negative control)		Relative population growth (% of negative control)		Cloning efficiency (%)		Mutant Frequency ($\times 10^{-6}$)	
Culture ID	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Experiment 1 (5 hour exposure)								
Untreated	112.4	101.8	129.7	97.9	78.7 ± 2.6	80.2 ± 3.8	1.1	9.4
0	100.0	100.0	100.0	100.0	87.0 ± 1.8	91.3 ± 1.6	5.3	10.0
46	119.0	94.6	128.4	106.0	75.3 ± 1.6	81.8 ± 1.0	0.0	5.1
92	125.9	99.5	112.2	107.8	73.3 ± 1.3	81.7 ± 1.8	14.2	4.6
184	112.1	120.5	120.8	120.6	74.3 ± 3.9	78.7 ± 3.0	6.2	10.6
368	94.5	110.9	80.6	135.7	82.5 ± 8.5	74.3 ± 4.6	1.0	0.0
736	122.3	119.7	119.8	122.9	87.2 ± 3.8	83.7 ± 8.0	5.7	4.5
1472	107.4	128.2	114.5	144.4	79.2 ± 1.3	80.0 ± 5.3	3.7	3.6
2944[P]	106.9	49.2	101.8	68.8	73.2 ± 4.1	79.0 ± 4.5	5.1	7.4
EMS	104.7	79.5	46.8	33.9	65.5 ± 8.8	71.7 ± 2.8	628.5	751.7
Experiment 2 (5 hour exposure)								
Untreated	99.1	8.26	62.2	103.3	83.2 ± 1.3	78.7 ± 4.9	2.0	6.4
0	100.0	100.0	100.0	100.0	75.7 ± 4.9	76.0 ± 4.3	2.2	4.9
46	91.8	90.2	58.7	91.0	76.7 ± 6.4	74.5 ± 3.1	6.0	5.6
92	90.3	93.9	55.3	97.4	74.3 ± 0.6	71.8 ± 1.9	1.7	4.6

Dose level (µg/mL)	Relative survival to treatment (% of negative control)		Relative population growth (% of negative control)		Cloning efficiency (%)		Mutant Frequency ($\times 10^{-6}$)	
Culture ID	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
184	93.8	84.4	54.5	94.9	81.2 ± 5.0	74.3 ± 4.3	8.7	9.5
368	82.6	71.3	54.9	84.9	74.5 ± 5.2	77.8 ± 3.3	4.5	1.6
736	96.1	72.4	56.2	75.8	82.5 ± 4.3	75.7 ± 4.5	1.5	4.4
1472	91.2	77.6	63.3	87.8	72.8 ± 4.4	75.2 ± 5.1	4.6	3.9
2944[P]	90.6	77.4	75.0	60.1	64.3 ± 6.6	80.0 ± 1.3	1.9	6.8
EMS	40.1	43.9	19.9	37.0	75.2 ± 6.8	79.2 ± 4.6	680.7	495.8

Survival to treatment = mean number of colonies (treated cultures) ÷ mean number of colonies (negative control). Mutant frequency = # of 6-TG resistant mutants per 10^6 clonable cells. [P] = precipitation observed.

Table 2: HPRT test outcomes after treatment of V79 cells with BYI 02960 (+S9 metabolic activation)

Dose level (µg/mL)	Relative survival to treatment (% of negative control)		Relative population growth (% of negative control)		Cloning efficiency (%)		Mutant Frequency ($\times 10^{-6}$)	
Culture ID	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Experiment 1 (5 hour exposure)								
Untreated	105.4	80.8	128.3	130.0	64.3 ± 3.3	69.7 ± 4.3	9.7	0.6
0	100.0	100.0	100.0	100.0	68.8 ± 3.5	77.3 ± 1.9	1.2	1.4
46	97.9	96.1	106.1	127.0	70.3 ± 8.2	76.7 ± 6.2	1.8	4.9
92	82.5	92.0	89.4	124.1	78.3 ± 4.3	76.7 ± 4.4	1.6	C
184	89.6	81.0	103.4	137.0	72.5 ± 2.2	73.0 ± 7.8	2.3	9.7
368	104.8	89.8	127.7	134.1	81.0 ± 4.8	73.3 ± 7.3	3.1	5.7
736	106.9	93.1	111.7	102.2	71.2 ± 9.2	83.8 ± 2.1	9.4	6.5
1472	96.3	99.1	90.4	137.5	80.7 ± 5.9	80.8 ± 4.8	8.3	8.2
2944[P]	90.8	NS	98.3	4.6	76.7 ± 6.2	NS	2.7	NS
DMBA	101.0	55.9	57.1	22.4	79.7 ± 8.1	84.5 ± 4.9	161.6	150.4

Dose level (µg/mL)	Relative survival to treatment (% of negative control)		Relative population growth (% of negative control)		Cloning efficiency (%)		Mutant Frequency ($\times 10^{-6}$)	
Culture ID	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Experiment 2 (5 hour exposure)								
Untreated	87.0	88.8	105.4	78.1	68.2 ± 6.4	83.5 ± 1.8	0.6	0.5
0	100.0	100.0	100.0	100.0	93.8 ± 9.5	78.0 ± 4.3	0.9	2.1
46	94.6	76.8	114.3	49.1	83.3 ± 9.5	82.5 ± 10.0	2.0	0.5
92	99.4	86.2	94.4	55.7	82.0 ± 3.9	67.0 ± 3.0	2.0	0.0
184	92.4	107.2	114.0	45.8	80.8 ± 2.5	74.7 ± 7.5	2.6	1.1
368	99.2	106.1	99.3	67.4	78.5 ± 3.5	79.8 ± 6.1	0.5	1.6
736	105.0	104.4	85.8	38.3	T	80.7 ± 9.0	T	1.0
1472	109.3	100.0	116.6	33.6	82.0 ± 3.8	93.5 ± 10.1	0.0	0.9
2944[P]	94.0	89.5	82.4	48.8	76.0 ± 1.3	85.7 ± 1.4	2.7	0.5
DMBA	36.7	18.6	22.8	12.6	76.0 ± 5.3	79.5 ± 7.8	121.7	139.9

Survival to treatment = mean number of colonies (treated cultures) ÷ mean number of colonies (negative control). Mutant frequency = # of 6-TG resistant mutants per 10^6 clonable cells. T = interim termination due to lack of surviving cells. C = contamination of cells, no scoring conducted. NS = not cloned for scoring. [P] = precipitation observed.

Primary review: Australis
Secondary review: US EPA& PMRA

B.6.8.3/12 *In vitro* genotoxicity: Test for gene mutation in mammalian cells

Report:	Hall, C. (2010a); BCS-AA56716 (metabolite of BYI 02960): Gene Mutation Assay in Chinese Hamster V79 cells *In Vitro* (V79/HPRT). Harlan CCR, Rossdorf, Germany. Study ID: 1351102. Ref: M-409727-01-02. MRID 48844129. Unpublished.
Dates of work:	27 July – 10 September 2010. Final report 20 December 2010
Guidelines:	US EPA OPPTS 870.5300 (1998); EC 440/2008, B17 (2008); OECD TG 476 (1998)
GLP/QA:	OECD, US EPA, EC and JMAFF GLP-compliant Signed and dated Quality Assurance and Data Confidentiality were presented in the report.

Executive summary:

In a Chinese Hamster V79 cell/HPRT mutation assay (Ref: M-409727-01-02), BCS-AA56716 (difluoroacetic acid) (99.6% purity) was tested in the presence and absence of an exogenous metabolic activation system (phenobarbital/β-naphthoflavone-induced rat liver S9) for its potential to cause gene mutation at the HPRT locus. Following a preliminary toxicity assay, duplicate flasks of exponentially growing V79 cells were exposed for 4 hours at 37°C to the test substance at concentrations of 0, 30, 60, 120, 240, 480 and 960 µg/mL with and without metabolic activation, and to the same concentrations for 24 hours at 37°C without metabolic activation. The highest concentration level was set based on the lack of test substance precipitation at a concentration equivalent to the limit dose of approximately 10 mM. Cells were then independently subcultured for assessment of cytotoxicity (cloning efficiency) and for expression and selection of the 6-thioguanine (2-amino-6-mercaptopurine)-resistant phenotype. The test substance was dissolved in dimethyl sulfoxide (DMSO), with ethyl methanesulfonate (EMS) and dimethylbenzanthracene (DMBA) used as positive controls for the non-activated and activated test systems, respectively.
In the mutagenesis assay, no evidence of precipitate formation was observed at the limit dose of 960 µg/mL. No excessive cytotoxicity was observed at any concentration tested, and no biologically relevant or treatment-related increases in mutant frequency were observed.
BCS-AA56716 was negative in the non-activated and S9-activated test systems in the V79/HPRT mutation assay.
This study is reliable (acceptable/guideline) and satisfies the requirements for an *in-vitro* mammalian cell gene mutation assay (OPPTS 870.5300; OECD 476).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BCS-AA56717
   Lot/Batch #: BCOO 5984-4-11
   Purity: 99.6%
   Description: Colourless liquid (from Certificate of Analysis).
   CAS #: 381-73-7
   Stability of test compound: No pre-experiment stability/solubility testing was conducted. Osmolarity and pH values were recorded in solvent control and the top dose of 960 µg/mL, which indicated no treatment-related differences in these parameters, suggesting test compound stability under the conditions of the study.
2. Control materials
   Solvent control: Dimethyl sulfoxide (DMSO)
   Positive, non-activation: Ethyl methanesulfonate (EMS) at a final concentration of 150 µg/mL (1.2 mM)
   Positive, activation: Dimethylbenzanthracene (DMBA) in DMSO at a final concentration of 1.1 µg/mL (4.3 µM)
3. Activation: Rat liver S9 from Male Wistar rats (phenobarbital/β-naphthoflavone-induced)
   Lot number: 160401 (preliminary experiment); 210510 (Main experiments)
   Source: In-house preparation
   Protein content: 35.0 mg/mL (preliminary experiment); 33.7 mg/mL (Main experiments)
   Characterisation: The metabolic activation ability of the S9 was assayed for its metabolising capacity using 2-aminoanthracene and benzo(a)pyrene.

   S9 mix composition
   NADP: 4 mM
   Glucose-6-phosphate: 5 mM
   KCl: 33 mM
   $MgCl_2$: 8 mM
   Na phosphate buffer (pH 7.4): 100 mM
   S9 homogenate: 0.75 mg/mL final concentration
4. Test cells

Chinese hamster V79 cells were obtained from LMP Technical University (Darmstadt, Germany). Karyotype and mycoplasma contamination of the V79 cells was checked prior to freezing and storage, with suppression of spontaneous mutants by treatment with HAT-mediuim.

5. Culture medium

Minimal Essential Media (MEM), supplemented with Hank's salts, neomycin (5 μg/mL) and amphotericin B (1 %) was used during seeding and treatment of cell cultures. Where appropriate, 50 μL of S9 mix was used in S9-activated assays.

6. Locus examined

Hypoxanthine-guanine-phosphoribosyl transferase (HPRT) – the selection agent used was 6-thioguanine (6TG, 2-amino-6-mercaptopurine) at a final concentration of 11 μg/mL in culture.

7. Test compound concentrations used

Preliminary cytotoxicity assay:	Concentrations of 0, 7.3, 14.7, 29.4, 58.9, 117.5, 235, 420 and 940 μg/mL test material were evaluated in the presence and absence of S9 activation. Exposure periods were 4 hours (±S9), with an additional separate experiment series with a 24-hour exposure period (–S9 only).
Mutagenesis assay:	Concentrations of 0, 30, 60, 120, 240, 480 and 960 μg/mL test material were evaluated in the presence and absence of S9 activation. Exposure periods were 4 hours (±S9; designated Experiment I), with an additional separate experiment series (designated Experiment II) with a 24-hour exposure period (–S9 only) and a 4-hour exposure period (+S9)

B. STUDY DESIGN AND METHODS

1. Experimental start/completion:

27 July – 10 September 2010

2. Preliminary cytotoxicity assay

V79 cells were exposed for 4 hours to vehicle alone and eight concentrations of test substance ranging from 7.3–940 μg/mL (equivalent to 9.8 mM, just below the limit dose) in both the absence and presence of S9-activation for evaluation of test substance effect on relative cloning efficiency (CE). An additional series was established where cells were exposed to the same concentrations of test substance for 24 hours without S9 activation.

3. Mutagenesis assay

V79 cells were exposed for 4 hours to the vehicle alone, appropriate positive controls and six concentrations of test substance in duplicate in both the absence and presence of S9-activation at 37°C in a 1.5% CO_2/98.5% air atmosphere. After 4 hours, the cells were washed with "Saline G" (8 g/L NaCL, 0.4 g/L KCl, 1.1 g/L glucose, 0.192 g/L $Na_2HPO_4.2H_2O$ and 0.15 g/L KH_2PO_4; pH 7.2), trypsinised and re-plated in culture

medium (MEM as described above, supplemented with 10% FCS) for cytotoxicity and phenotypic expression/selection evaluation (see below).

Similarly, V79 cells were exposed for 24 hours to the vehicle alone, appropriate positive controls and six concentrations of test substance in duplicate in the absence of S9 activation at 37°C in a 1.5% CO_2/98.5% air atmosphere, and processed at the end of the exposure period as described above for 4-hour exposure groups.

Cells were then cultured to determine cell cytotoxicity (cloning efficiency I), viability (cloning efficiency II) and phenotypic expression/selection as described below:

Cell survival/viability (relative cloning efficiency I, relative cell density, and relative cloning efficiency II): Three to four days after treatment, 1.5×10^6 cells per dose level were sub-cultured in flask form. Seven days after treatment (i.e. the end of the expression time), duplicate flasks were seeded with ~500 cells each in non-selective medium to determine cell viability. Cultures were incubated for an additional 8 days prior to staining with 10 % methylene blue in 0.01% KOH solution.

Phenotypic expression/selection: Three to four days after treatment, 1.5×10^6 cells per dose level were sub-cultured in flask form. Seven days after treatment (i.e. the end of the expression time), cultures were re-seeded ($3–5\times10^5$ cells) into five separate flasks per dose level into medium containing 6-TG for mutant selection. Cultures were incubated for an additional 8 days prior to staining with 10 % methylene blue in 0.01% KOH solution.

4. Statistics

A least squares linear regression analysis was performed to determine whether a dose-dependent increase in mutant frequencies occurred, with a trend significance threshold of $p < 0.05$.

5. Evaluation criteria

The study was considered valid and acceptable if the following criteria were met:

- The mutant frequency (number of mutant colonies per 10^6 cells) found in solvent controls were within laboratory historical controls
- The mutant frequency found in positive controls was significantly increased (interpretation guided by historical control data)
- The absolute cloning efficiency for solvent controls was > 50%.

The study results were interpreted as follows:

- A positive result was defined as a positive response (i.e. increased mutant frequency) at one of the test concentrations, where the increase was ≥3× the spontaneous mutant frequency, or where a reproducible concentration-related increase in the mutant frequency was observed, with biological relevance considered where the 3× increase in spontaneous mutant frequency was not achieved.

- A negative result was defined as the absence of a reproducible or concentration-related increase in mutant frequency.

The criteria above could be over-ruled with good scientific judgement, including consideration of any unusual variation in the concurrent solvent controls (compared with historical control data).

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

DMSO was determined to be the solvent of choice based on the solubility of the test substance and compatibility with the target cells. In preliminary experiments where cells were exposed to test substance for 4 hours (±S9) and 24 hours (–S9), no precipitation of test material or phase separation was observed, and no cytotoxic effects were observed at up to 940 µg/mL (approaching the limit dose of 10 mM).

B. MUTAGENESIS ASSAY

The results from the main study assay are summarized in Tables 1 and 2. No test substance precipitation was observed at all doses tested, and no significant cytotoxicity (defined as a relative cloning efficiency I or cell density of 10–20%) occurred in cultures. However, moderate cytotoxicity (reported as up to 60% decreases in relative cell density) were observed in both sets of cultures (±S9) used in Experiment II.

Across the two experiments, no treatment-related increase in mutant frequency was observed at up to (and including) the limit dose. Overall, the mutant frequency range for concurrent negative controls was 5.8–45.3 (per 10^6 cells), while mutant frequencies from cultures treated with the test substance were between 8.6–54.4 (per 10^6 cells). Positive controls elicited the expected increase in mutant frequency.

There were notable (i.e. ≥3×) increases in mutant colonies compared with concurrent negative controls in the first culture of Experiment I without metabolic activation suggesting a positive response (as defined by the laboratory evaluation criteria). However, the low frequency of spontaneous mutant colonies in the concurrent negative control (mutant frequency of 5.8; compared with a mean value of 14 ± 6.6), and the lack of reproducibility for this effect in the second parallel culture in Experiment I (mutant frequency of 24.8, and treated cells reporting similar mutant frequencies to those observed in the first culture) suggest that the apparent positive response elicited in treated cells in the first culture of Experiment I is not biologically relevant in this case.

Statistical analyses did not report a treatment-related dose dependency in mutation frequency.

Table 1: HPRT test outcomes after treatment of V79 cells with BCS-AA56717 (–S9 metabolic activation)

Dose level (µg/mL)	Relative Cloning efficiency I (% of negative control)	Relative population growth [cell density] (% of negative control)	Relative Cloning efficiency II (% of negative control)	Mutant Frequency ($\times 10^{-6}$)

Culture ID	A	B	A	B	A	B	A	B
Experiment 1 (4 hour exposure)								
0	100.0	100.0	100.0	100.0	100.0	100.0	5.8	24.8
30	70.8	60.7	NR	NR	NR	NR	NR	NR
60	87.7	89.4	87.0	92.0	83.3	90.1	34.5	22.8
120	92.1	77.1	71.2	89.6	88.5	93.8	38.5	35.5
240	100.2	92.0	84.8	114.9	91.9	89.5	27.2	17.1
480	87.7	98.8	60.4	115.8	103.8	76.3	38.3	16.4
960	82.2	95.9	101.8	126.0	96.4	64.1	24.0	22.1
EMS (150)	79.9	75.4	81.2	116.3	84.3	77.1	268.6	168.9
Experiment 2 (24 hour exposure)								
0	100.0	100.0	100.0	100.0	100.0	100.0	38.4	14.5
30	84.3	91.2	81.0	99.8	106.4	95.5	28.8	13.7
60	101.2	104.2	82.3	103.7	111.2	95.7	22.7	23.4
120	95.2	85.6	93.6	107.0	101.7	89.7	22.3	11.8
240	94.9	97.0	88.1	79.6	95.7	96.7	25.3	28.2
480	86.4	87.0	C	C	C	C	C	C
960	83.6	75.0	68.0	41.7	102.7	94.8	22.3	22.1
EMS (150)	90.2	102.6	88.7	100.0	81.7	91.5	534.4	410.3

Mutant frequency = # of 6-TG resistant mutants per 10^6 clonable cells. NR = not required (sufficient analysable concentrations). C = contaminated culture.

Table 2: HPRT test outcomes after treatment of V79 cells with BCS-AA56717 (+S9 metabolic activation)

Dose level (µg/mL)	Relative survival to treatment (% of negative control)		Relative population growth (% of negative control)		Cloning efficiency (%)		Mutant Frequency ($\times10^{-6}$)	
Culture ID	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Experiment 1 (4 hour exposure)								
0	100.0	100.0	100.0	100.0	100.0	100.0	45.3	25
30	108.8	87.7	NR	NR	NR	NR	NR	NR
60	97.2	86.3	90.0	129.9	87.8	101.9	18.4	54
120	98.5	93.5	85.3	125.4	102.8	107.5	8.6	21
240	104.9	92.6	97.9	112.6	90.8	106.7	20.9	37
480	105.4	86.1	97.5	111.4	97.5	114.8	34.5	10
960	110.1	82.4	104.9	105.6	87.1	92.4	41.1	35
DMBA (1.1)	33.6	38.1	67.9	97.6	82.8	96.8	1232.2	100
Experiment 2 (4 hour exposure)								
0	100.0	100.0	100.0	100.0	100.0	100.0	24.5	27
30	99.0	85.2	NR	NR	NR	NR	NR	NR
60	105.4	86.4	NR	NR	NR	NR	NR	NR
120	110.4	87.0	98.1	66.2	105.2	100.3	23.1	24
240	102.8	94.8	139.2	56.4	100.3	99.3	26.5	37
480	112.0	87.6	144.1	47.8	101.7	95.0	52.8	27
960	115.3	100.7	104.5	51.0	103.0	96.6	33.9	27
DMBA (1.1)	27.0	21.2	44.2	37.2	70.3	58.2	1337.5	118

Mutant frequency = # of 6-TG resistant mutants per 10^6 clonable cells. NR = not required (sufficient analysable concentrations). C = contaminated culture.

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 476 (1997) and other internationally-recognised equivalent test guidelines.

C. CONCLUSIONS: BCS-AA56717 was negative for mutagenic activity up to the limit dose of 960 μg/mL (equivalent to 10 mM) in both trials in V79 Chinese Hamster cells in the presence and absence of S9-activation in the *in vitro* mammalian cell gene mutation assay. There were no treatment-related increases in mutation frequencies. The positive controls induced marked increases in revertant colonies compared to controls in the presence and absence of S9 activation.

Accordingly, BCS-AA56717 is negative in this test system for mutagenicity.

Primary review: Australia
Secondary review: US EPA & PMRA

B.6.8.3/13 *In vitro* genotoxicity: Test for gene mutation in mammalian cells

Report: Wollny, H-E (2011); BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960): Gene Mutation Assay in Chinese Hamster V79 cells *In Vitro* (V79/HPRT). Harlan CCR, Rossdorf, Germany. Study ID: 1399702. Ref: M-420095-01-2. MRID 48844130. Unpublished.

Dates of work: 17 March – 8 July 2011. Final report 20 December 2011

Guidelines: US EPA OPPTS 870.5300 (1998); EC 440/2008, B17 (2008); OECD TG 476 (1998)

GLP/QA: OECD, US EPA, EC and JMAFF GLP-compliant
Signed and dated Quality Assurance and Data Confidentiality were presented in the report.

Executive summary:

In a Chinese Hamster V79 cell/HPRT mutation assay (Ref: M-420095-01-2), BYI 02960-difluoroethyl-amino-furanone (98.5% purity) was tested in the presence and absence of an exogenous metabolic activation system (phenobarbital/β-naphthoflavone-induced rat liver S9) for its potential to cause gene mutation at the HPRT locus. Following a preliminary toxicity assay, duplicate flasks of exponentially growing V79 cells were exposed for 5 hours at 37°C to the test substance at concentrations of 0, 51.3, 102.5, 205, 410, 820 and 1640 μg/mL, and to the same concentrations for 24 hours at 37°C without metabolic activation. The highest concentration level was set based on the lack of test substance precipitation at the limit dose of 10 mM. Cells were then independently subcultured for assessment of cytotoxicity (cloning efficiency) and for expression and selection of the 6-thioguanine (2-amino-6-mercaptopurine)-resistant phenotype. The test substance was dissolved in dimethyl sulfoxide (DMSO), with ethyl methanesulfonate (EMS) and dimethylbenzanthracene (DMBA) used as positive controls for the non-activated and activated test systems, respectively.
In the mutagenesis assay, no test substance precipitate was observed at the limit dose of 1640 μg/mL. No excessive cytotoxicity was observed at any concentration tested, and no biologically relevant or treatment-related increases in mutant frequency were observed.
BYI 02960-difluoroethyl-amino-furanone was negative in the non-activated and S9-activated test systems in the V79/HPRT mutation assay.
This study is reliable (acceptable/guideline) and satisfies the requirements for an *in-vitro* mammalian cell gene mutation assay (OPPTS 870.5300; OECD 476).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960-difluoroethyl-amino-furanone
   Lot/Batch #: NLL 8671-6-1
   Purity: 98.5%
   Description: White powder
   CAS #: Not specified
   Stability of test compound: No pre-experiment stability/solubility testing was conducted. Osmolarity and pH values were recorded in solvent control and the top dose of 1640 µg/mL, which indicated no treatment-related differences in these parameters, suggesting test compound stability under the conditions of the study.
2. Control materials
   Solvent control: Deionised water
   Positive, non-activation: Ethyl methanesulfonate (EMS) in culture medium at a final concentration of 150 µg/mL (1.2 mM)
   Positive, activation: Dimethylbenzanthracene (DMBA) in DMSO at a final concentration of 1.1 µg/mL (4.3 µM)
3. Activation: Rat liver S9 from Male Wistar rats (phenobarbital/β-naphthoflavone-induced)
   Lot number: 291010 (preliminary experiment + Experiment I); 150411 (Experiment 2)
   Source: In-house preparation
   Protein content: 35.2 mg/mL (preliminary experiment + Experiment I); 32.6 mg/mL (Experiment 2)
   Characterisation: Not specified
   S9 mix composition
   NADP: 4 mM
   Glucose-6-phosphate: 5 mM
   KCl: 33 mM
   $MgCl_2$: 8 mM
   Na phosphate buffer (pH 7.4): 100 mM
   S9 homogenate: 0.75 mg/mL final concentration
4. Test cells

Chinese hamster V79 cells were obtained from LMT, Technical University (Darmstadt, Germany). Karyotype and mycoplasma contamination of the V79 cells was checked prior to freezing and storage, with suppression of spontaneous mutants by treatment with HAT-mediuim.

5. Culture medium

Minimal Essential Media (MEM), supplemented with Hank's salts, neomycin (5 μg/mL) and amphotericin B (1 %) was used during seeding and treatment of cell cultures. Where appropriate, 50 μL of S9 mix was used in S9-activated assays.

6. Locus examined

Hypoxanthine-guanine-phosphoribosyl transferase (HPRT) – the selection agent used was 6-thioguanine (6TG, 2-amino-6-mercaptopurine) at a final concentration of 11 μg/mL in culture.

7. Test compound concentrations used

Preliminary cytotoxicity assay:	Concentrations of 0, 12.8, 25.6, 51.3, 102.5, 205, 410, 820, 1640 μg/mL test material were evaluated in the presence and absence of S9 activation. Exposure periods were 4 hours (±S9), with an additional separate experiment series with a 24-hour exposure period (–S9 only).
Mutagenesis assay:	Concentrations of 0, 51.3, 102.5, 205, 410, 820 and 1640 μg/mL test material were evaluated in the presence and absence of S9 activation. Exposure periods were 4 hours (±S9; designated Experiment I), with an additional separate experiment series (designated Experiment II) with a 24-hour exposure period (–S9 only) and a 4-hour exposure period (+S9)

B. STUDY DESIGN AND METHODS

1. Experimental start/completion:

17 March – 8 July 2011

2. Preliminary cytotoxicity assay

V79 cells were exposed for 4 hours to vehicle alone and eight concentrations of test substance ranging from 12.8–1640 μg/mL (equivalent to 10 mM, the limit dose) in both the absence and presence of S9-activation for evaluation of test substance effect on relative cloning efficiency (CE). An additional series was established where cells were exposed to the same concentrations of test substance for 24 hours without S9 activation.

3. Mutagenesis assay

V79 cells were exposed for 4 hours to the vehicle alone, appropriate positive controls and six concentrations of test substance in duplicate in both the absence and presence of S9-activation at 37°C in a 1.5% CO_2/98.5% air atmosphere. After 4 hours, the cells were washed with "Saline G" (8 g/L NaCL, 0.4 g/L KCl, 1.1 g/L glucose, 0.192 g/L $Na_2HPO_4.2H_2O$ and 0.15 g/L KH_2PO_4; pH 7.2), trypsinised and re-plated in culture

medium (MEM as described above, supplemented with 10% FCS) for cytotoxicity and phenotypic expression/selection evaluation (see below).

Similarly, V79 cells were exposed for 24 hours to the vehicle alone, appropriate positive controls and six concentrations of test substance in duplicate in the absence of S9 activation at 37°C in a 1.5% CO_2/98.5% air atmosphere, and processed at the end of the exposure period as described above for 4-hour exposure groups.

Cells were then cultured to determine cell viability and phenotypic expression/selection as described below:

Cytotoxicity/cell viability: Three to four days after treatment, 1.5×10^6 cells per dose level were sub-cultured in flask form. Seven days after treatment (i.e. the end of the expression time), duplicate flasks were seeded with ~500 cells each in non-selective medium to determine cell viability. Cultures were incubated for an additional 8 days prior to staining with 10 % methylene blue in 0.01% KOH solution.

Phenotypic expression/selection: Three to four days after treatment, 1.5×10^6 cells per dose level were sub-cultured in flask form. Seven days after treatment (i.e. the end of the expression time), cultures were re-seeded ($3–5\times10^5$ cells) into five separate flasks per dose level into medium containing 6-TG for mutant selection. Cultures were incubated for an additional 8 days prior to staining with 10 % methylene blue in 0.01% KOH solution.

4. Statistics

A least squares linear regression analysis was performed to determine whether a dose-dependent increase in mutant frequencies occurred, with a trend significance threshold of $p < 0.05$.

5. Evaluation criteria

The study was considered valid and acceptable if the following criteria were met:

- The mutant frequency (number of mutant colonies per 10^6 cells) found in solvent controls were within laboratory historical controls
- The mutant frequency found in positive controls was significantly increased (interpretation guided by historical control data)
- The absolute cloning efficiency for solvent controls was > 50%.

The study results were interpreted as follows:

- A positive result was defined as either a positive response (i.e. increased mutant frequency) at one of the test concentrations, where the increase was ≥3× the spontaneous mutant frequency, or where a reproducible concentration-related increase in the mutant frequency was observed, with biological relevance considered where the 3× increase in spontaneous mutant frequency was not achieved.
- A negative result was defined as the absence of a reproducible or concentration-related increase in mutant frequency.

The criteria above could be over-ruled with good scientific judgement, including consideration of any unusual variation in the concurrent solvent controls (compared with historical control data).

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

DMSO was determined to be the solvent of choice based on the solubility of the test substance and compatibility with the target cells. In preliminary experiments where cells were exposed to test substance for 4 hours (±S9) and 24 hours (–S9), no precipitation of test material or phase separation was observed, and no cytotoxic effects were observed at up to 1640 μg/mL (approaching the limit dose of 10 mM).

B. MUTAGENESIS ASSAY

The results from the main study assay are summarized in Tables 1 and 2. No test substance precipitation was observed at all doses tested, and no significant cytotoxicity (defined as a relative cloning efficiency I or cell density of 10–20%) occurred in cultures. Across the two experiments, no treatment-related increase in mutant frequency was observed at up to (and including) the limit dose. Overall, the mutant frequency range for concurrent negative controls was 6.4–30.0 (per 10^6 cells), while mutant frequencies from cultures treated with the test substance were between 5.7–33.2 (per 10^6 cells). Positive controls elicited the expected increase in mutant frequency.

There were notable (i.e. ≥3×) increases in mutant colonies compared with concurrent negative controls at 410 and 820 μg/mL in the first culture of Experiment I without metabolic activation (4-hour exposure) suggesting a positive response (as defined by the laboratory evaluation criteria). However, the observed increase was not considered biologically relevant due to the lack of reproducibility in the second culture, the similarity of the reported mutant frequencies to laboratory historical controls, and the lack of a dose response (noting that mutant frequencies at the limit dose were below the 3× concurrent solvent control threshold for a positive response).

Statistical analyses reported a significant increasing dose-related trend in mutation frequency in the first culture of Experiment II without metabolic activation (24-hour exposure), and in the second culture of Experiment II with metabolic activation (4-hour exposure). However, as all results were within ranges expected in both concurrent and laboratory historical controls, and were not a reproducible effect in the parallel cultures in the same experimental series, the statistical significance was not considered biologically relevant.

Table 1: HPRT test outcomes after treatment of V79 cells with BYI 02960-difluoroethyl-amino-furanone (–S9 metabolic activation)

Dose level (µg/mL)	Relative Cloning efficiency I (% of negative control)		Relative population growth [cell density] (% of negative control)		Relative Cloning efficiency II (% of negative control)		Mutant Frequency ($\times 10^{-6}$)	
Culture ID	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Experiment 1 (4 hour exposure)								
0	100.0	100.0	100.0	100.0	100.0	100.0	6.4	9.6
51.3	98.3	99.6	NR	NR	NR	NR	NR	NR
102.5	97.1	98.1	102.9	123.9	109.8	101.0	5.7	11.5
205	91.7	97.1	109.6	116.4	99.7	90.3	13.5	13.7
410	97.1	98.5	107.9	120.2	69.8	100.1	26.8	6.4
820	94.7	96.7	108.1	118.4	76.7	72.8	19.8	11.0
1640	100.4	96.2	110.8	111.7	75.5	92.0	15.2	16.4
EMS (150)	51.2	50.1	99.5	117.5	95.1	117.4	100.4	68.7
Experiment 2 (24 hour exposure)								
0	100.0	100.0	100.0	100.0	100.0	100.0	17.5	30.0
51.3	88.6	97.8	NR	NR	NR	NR	NR	NR
102.5	91.3	94.2	95.5	106.9	86.3	83.7	15.9	21.1
205	96.3	91.0	101.1	101.9	154.5	84.8	16.4	21.5
410	90.2	93.0	101.7	86.9	116.0	91.3	22.3	16.9
820	96.0	91.0	92.2	86.1	107.3	85.2	16.7	16.7
1640	106.2	103.6	109.7	93.8	159.9	79.9	31.6	22.3
EMS (150)	91.8	82.6	95.5	86.7	114.1	81.7	372.5	381.8

Mutant frequency = # of 6-TG resistant mutants per 10^6 clonable cells. NR = not required (sufficient analysable concentrations).

Table 2: HPRT test outcomes after treatment of V79 cells with BYI 02960-difluoroethyl-amino-furanone (+S9 metabolic activation)

Dose level (µg/mL)	Relative survival to treatment (% of negative control)		Relative population growth (% of negative control)		Cloning efficiency (%)		Mutant Frequency ($\times 10^{-6}$)	
Culture ID	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Experiment 1 (4 hour exposure)								
0	100.0	100.0	100.0	100.0	100.0	100.0	7.4	18.9

51.3	94.6	99.5	NR	NR	NR	NR	NR	NR
102.5	100.0	92.2	90.6	97.0	92.0	96.2	20.7	11.2
205	96.6	104.6	100.9	82.4	92.7	94.6	17.9	15.7
410	95.0	95.8	101.2	72.0	90.9	114.1	18.0	9.5
820	98.0	99.0	101.3	97.4	92.0	90.2	10.5	15.9
1640	97.2	101.6	80.6	101.0	87.3	130.5	14.1	8.1
DMBA (1.1)	36.7	46.3	45.0	41.6	81.4	103.1	752.9	640.2
Experiment 2 (4 hour exposure)								
0	100.0	100.0	100.0	100.0	100.0	100.0	24.2	8.2
51.3	107.9	113.1	NR	NR	NR	NR	NR	NR
102.5	100.9	107.0	100.4	110.1	93.7	80.2	14.3	11.6
205	99.9	111.6	92.4	91.1	95.2	70.0	31.9	11.6
410	103.2	113.6	126.1	94.6	93.1	76.5	33.2	11.8
820	95.3	104.5	112.2	97.9	85.1	72.5	21.8	12.4
1640	97.0	114.7	125.8	109.7	92.8	73.5	27.4	15.0
DMBA (1.1)	48.5	48.7	47.2	44.8	65.9	46.5	773.8	596.2

Mutant frequency = # of 6-TG resistant mutants per 10^6 clonable cells. NR = not required (sufficient analysable concentrations).

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 476 (1997) and other internationally-recognised equivalent test guidelines.

C. CONCLUSIONS: BYI 02960-difluoroethyl-amino-furanone was negative for mutagenic activity up to the limit dose of 1640 µg/mL (equivalent to 10 mM) in both trials in V79 Chinese Hamster cells in the presence and absence of S9-activation in the *in vitro* mammalian cell gene mutation assay. There were no treatment-related increases in mutation frequencies. The positive controls induced marked increases in revertant colonies compared to controls in the presence and absence of S9 activation.

Accordingly, BYI 02960-difluoroethyl-amino-furanone is negative in this test system for mutagenicity.

Primary review: Australia
Secondary Review: US EPA & PMRA

Report:	Thum, M. (2009). BYI 02960 (tested as BYI 02960 Technical) *In vitro* Chromosome Aberration Test with Chinese Hamster V79 Cells. Bayer Schering Pharma AG, Wuppertal, Germany. Report No.: AT05626. Study ID: T 9080065. Ref: M-359746-01-2. MRID 48844131. Unpublished.
Dates of work:	26 May – 5 October 2009. Final Report 11 November 2009.
Guidelines:	OPPTS 870.5375 (1998), ECC 2000/32/EC, B10 (2000), OECD No. 473 (1997).
GLP/QA:	OECD, US EPA, EC and JMAFF GLP-compliant Signed and dated Quality Assurance and Data Confidentiality statements were provided.

Executive Summary:

BYI 02960 (96.2%; Lot No. 2009-000239) was tested in the *in vitro* mammalian chromosome aberration test using Chinese Hamster V79 cells both with and without an exogenous metabolic activation system (Aroclor-induced rat liver S9). In a preliminary toxicity assay, the cells were exposed to nine concentrations of the test substance ranging from 0–3000 µg/mL (the limit dose) with a 4-hour treatment period, or seven concentrations from 0–3000 µg/mL with an 18-hour treatment period. The highest dose level tested in the main study was set based on production of cytotoxicity (or lack thereof where no treatment-related effects were observed at the limit dose) in this preliminary toxicity assay.

No test material precipitation was observed in the preliminary toxicity studies. No obvious changes in cell survival indices or mitotic indices were observed at ≤ 1000 µg/mL after a 4-hour exposure. A notable decrease in survival index (>35% decrease compared with solvent control; ±S9) and mitotic index (~30% decrease compared with solvent control (–S9) and ~12% decrease (+S9)) was observed at the limit dose of 3000 µg/mL (i.e. equivalent to 10mM). In cytotoxicity examination of cells treated with the test material for 18 hours without metabolic activation, a steady dose-related decrease in both survival index and mitotic index was observed.

In the main study, concentrations of 0, 500, 1000 and 2500 ug/ml BYI 02960 with 4 hours treatment and 18 hours harvest, and 0, 200, 400 and 800 ug/ml with 18 hours treatment and harvest were chosen in the absence of metabolic activation for reading. Additionally, cultures treated with 0 and 2500 ug/ml for 4 hours in the absence of metabolic activation were harvested at 30 hours were and read. In the presence of metabolic activation, cells were treated with 0, 500, 1000 and 3000 ug/ml BYI 02960 for 4 hours and harvested at 18 hours were chosen for reading. Additionally, cultures treated with 0 and 3000 ug/ml for 4 hours in the presence of metabolic activation were harvested at 30 hours were read. No statistically significant or dose-related increase in the rate of chromosome aberrations (± gaps) was noted with test material treatment for 4 hours (±S9) or 18 hours (–S9) treatment. Positive controls elicited the expected increase in

metaphases with aberrations at the different treatment time points with and without metabolic activation.

Based on the findings of this study, BYI 02960 was concluded to be negative for the induction of structural and numerical chromosome aberrations in cultured Chinese Hamster V79 cells with and without an exogenous metabolic activation system.

This study is classified as **reliable (acceptable/guideline)** and satisfies the guideline requirement for an i*n vitro* mammalian cytogenetics (chromosome aberrations) assay (OPPTS 870.5375; OECD 473).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:
   Lot/Batch #:
   Purity:
   Description:
   CAS #
   Stability of test compound:

   BYI 02960 technical
   2009-000239
   96.2%
   Fine beige powder
   951659-40-8
   Results from stability testing indicated that the test material was stable for at least 24 hours at 0.01 and 300 mg/mL in DMSO solvent, covering the time from formulation through to last treatment.

2. Control materials
   Solvent control:
   Positive, non-activation:
   Positive, activation:

   Dimethyl sulfoxide (DMSO)
   Mitomycin C (MMC) in water at 0.1 µg/mL (4-hour treatment) or 0.03 µg/mL (18-hour treatment) without S9 activation
   Cyclophosphamide (CP) in water at 2 µg/mL with S9 activation

3. Activation:
   Lot number:
   Source:
   Protein content:
   Characterisation:

   Rat liver S9 from Male Sprague-Dawley rats (Aroclor 1254-induced)
   Not specified
   In-house production
   25.9 mg/mL
   The metabolic activation ability of the S9 was assayed for its metabolising capacity using 2 µg/mL cyclophosphamide.

   S9 mix composition (per 25 mL)

Glucose-6-phosphate:	38.0 mg
NADP:	78.8 mg
KCl:	61.5 mg
$MgCl_2$:	40.7 mg
S9:	40 % (v/v)
Sodium phosphate buffer (pH 7.4)	15 mL (100 mM)

4. Test cells

Chinese hamster V79 cells were obtained from Merck AG (8 November 1993), and were brought from frozen stock and cultured in media at 37°C prior to study commencement. Karyotype of the V79 cells was confirmed prior to use in the study.

5. Culture medium (for the assays)

PAA ready mix was used as culture medium (Eagle's MEM, supplemented with 1 % each of L-glutamine, MEM-vitamins, MEM NEAA, and penicillin/streptomycin), with the addition of either 2 or 10% foetal bovine serum (FBS)

6. Test compound concentrations evaluated:

Preliminary cytotoxicity test: 0, 1, 10, 25, 50, 100, 250, 1000 or 3000 µg/mL ±S9 (4-hour treatment) or 0, 10, 100, 500, 1000, 2000 or 3000 µg/mL –S9 (18-hour treatment). The top dose of 3000 µg/mL was noted by the evaluator to be equivalent to the limit dose of 10 mM.

Main chromosome aberration assay:

Non-activated 4-hour exposure group:	0, 500, 1000, 2000, 2500 or 3000 µg/mL
S9-activated 4-hour exposure group:	0, 500, 1000, 2000, 2500 or 3000 µg/mL
Non-activated 18-hour exposure group:	0, 100, 200, 400, 600 or 800 µg/mL.

All cell cultures were set up in duplicate for each dose level, and incubated at 37°C in a 5% CO_2/95% air atmosphere.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

26 May – 5 October 2009

2. Treatment:

Preliminary cytotoxicity assay: The toxicity test was performed to select concentrations for the chromosome aberration assay and consisted of an evaluation of test substance effect on mitotic index (MI). The cells were exposed to a dose series of solvent alone and nine concentrations of the test substance for 4 hours in both the presence and absence of S9 activation, or a dose series of solvent alone and six concentrations of the test substance for 18 hours continuously in the absence of S9 activation. Based on preliminary results, a dose series of solvent control and five concentrations of the test substance were used in the main assay (see Section I.A.6 above).

Cytogenetic assay: Independently performed experiments were conducted

a. Cell exposure time:

Non-activated:	4 and 18 h (separate series of cultures; see Section I.A.6 above)
Activated:	4 h (different dose series compared with non-S9 activated assays; see Section I.A.6 above)

b. Spindle inhibition:

Inhibition used/concentration:	Colcemid (2 mL of 40 µg/mL solution)
Administration time:	2 hours (before cell harvest)

c.

Cell harvest time after termination of treatment:	Test material	Positive control
4-hour treatment (±S9)	18 h (1st harvest, all dose levels)	18 h only (MMC, 0.1 µg/mL or CP, 2.0 µg/mL)
	30 h (2nd harvest, 0, 2000, 2500 and 3000 µg/mL only)	
18-hour treatment (–S9)	18 h harvest only (all dose levels)	18 h harvest (MMC; 0.03 µg/mL)

d. Details of slide preparation: Following the 2-hour colcemid treatment, cells were treated with hypotonic KCl (0.4% solution, 37°C), then centrifuged and the supernatant removed. Cells were then re-suspended with a 3:1 mixture of ethanol and acetic acid for 20 minutes, centrifuged and resuspended again in the same fixative mixture, then air-dried for two hours and stained with 3% Giemsa. Two slides per group per experiment were prepared.

e. Metaphase analysis

No. of cells examined per dose: 200 (100 per duplicate culture) were examined.
Minimum 100 metaphases scored.
Mitotic index count: 1000 cells per culture

Scored for structural?	×	Yes		No
Scored for numerical?	×	Yes, polyploidy cells		No
Coded prior to analysis?	×	Yes		No

f. Evaluation criteria: The test substance was considered to induce a positive response if the percentage of cells with aberrations were increased in a "relevant and statistically significant" manner. The test substance was considered to be non-clastogenic if no such increase was

observed at any time interval, or where any statistically significant findings were within historical negative controls. The test substance was considered to be equivocal for clastogenic findings if an increase in aberrant metaphases above the rate seen in historical negative controls was observed, but statistical significance was not achieved and results from the concurrent negative controls were not "closely related" to the treatment group results.

g. Statistical analysis: Statistical analysis of the mitotic index was conducted using the one-sided χ^2 test ($p<0.05$) where the mitotic index was reduced compared with the solvent control. Statistical analysis of the number of aberrant metaphase cells excluding gaps was performed using the one-sided χ^2 test ($p<0.05$) where the number of aberrations were above the solvent control.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

DMSO was determined to be the solvent of choice based on the solubility of the test substance and compatibility with the target cells. Stability testing indicated that the test material was stable at 0.01 and 300 mg/mL for 24 hours in DMSO at room temperature, with no change in nominal test material concentrations. No test material precipitation was observed in culture medium used during the preliminary tests.

B. PRELIMINARY CYTOTOXICITY ASSAY

In preliminary cytotoxicity examinations, no obvious changes in cell survival indices or mitotic indices were observed at ≤ 1000 µg/mL after a 4-hour exposure. A notable decrease in survival index (>35% decrease compared with solvent control; ±S9) and mitotic index (~30% decrease compared with solvent control (–S9) and ~12% decrease (+S9)) was observed at the limit dose of 3000 µg/mL. In cytotoxicity examination of cells treated with the test material for 18 hours without metabolic activation, a steady dose-related decrease in both survival index and mitotic index was observed (Table 1), with a peak decrease in cell survival at the limit dose of 3000 µg/mL, and a similar finding in mitotic nuclei at 2000 µg/mL and above.

Clear alterations in cell morphology and non-attached cells were observed at ≥ 1000 µg/mL (±S9) in 4-hour treatment groups, and at ≥ 2000 µg/mL in 18-hour treatment groups (–S9).

The main dose series were selected based on these preliminary results.

Table 1: Survival and Mitotic indices from preliminary cytotoxicity study with BYI 02960.

Treatment time	Dose level (µg/mL)	Mitotic nuclei (%)		Survival Index (%)	
		–S9	+S9	–S9	+S9
4 hours	0	100.0	100.0	100.0	100.0
	1	99.0	96.9	106.6	122.4
	10	78.4	88.0	80.8	106.8
	25	98.0	98.2	102.4	81.6
	50	93.6	119.6	91.0	104.1
	100	91.7	104.9	111.4	94.6
	250	89.7	91.6	100.0	112.2
	1000	80.9	82.7	86.2	100.7
	3000	69.6	88.4	46.1	65.3
18 hours	0	100.0	N/A	100.0	N/A
	10	105.2		105.4	
	100	106.9		98.5	
	500	62.9		80.0	
	1000	33.6		67.8	
	2000	6.9		58.0	
	3000	9.5		42.9	

N/A = NOT APPLICABLE (NOT MEASURED)

C. MAIN CHROMOSOME ABERRATION ASSAY

Summarized results from the chromosome aberration assays are presented in Tables 2–6 below and are as follows:

Without metabolic activation (–S9): The survival index for cells was notably decreased (at relevant levels) at ≥ 1000 µg/mL (4-hour treatment) and at ≥ 600 µg/mL (18-hour treatment). The positive control mitomycin C also elicited a decrease in survival index.

The mitotic index for cells was also notably decreased with treatment at ≥ 2000 µg/mL (4-hour treatment) and at ≥ 400 µg/mL (18-hour treatment). The positive control mitomycin C only elicited a decrease in mitotic index in the 4-hour treatment system without metabolic activation.

With metabolic activation (+S9): The survival index and mitotic index for cells was notably decreased (at relevant levels) at ≥ 2000 µg/mL. The positive control cyclophosphamide also elicited a decrease in survival index and mitotic index.

Based on the survival and mitotic index data from the main study, at least three relevant dose levels from the 4-hour treatment groups (0, 500, 1000 and 2500 µg/mL, –S9; 0, 500, 1000 and 3000 µg/mL, +S9) and the 18-hour treatment group (0, 200, 400 and 800 µg/mL, –S9) were selected for chromosome aberration screening. Results from chromosome aberration screening are shown in Tables 2–6 below. In all cases, no statistically significant or dose-related increase in the rate of chromosome aberrations (± gaps) was noted with test material treatment. Positive

controls elicited the expected increase in metaphases with aberrations at the different treatment time points with and without metabolic activation.

Table 2: Summary of main parameters from chromosome aberration study in V79 cells

Treatment time	Harvest Time	Dose level (µg/mL)	Mitotic nuclei per 2000 cells (%)		Survival Index (%)		Metaphases with aberrations (%)			
							–S9		+S9	
			–S9	+S9	–S9	+S9	Incl. gaps	Excl. gaps	Incl. gaps	Excl. gaps
4 hours	8	0	100.0	100.0	100.0	100.0	N/A			
	8	2000	65.7**	57.8	83.6	97.1				
	8	2500	44.1**	53.1	83.6	74.3#				
	8	3000	41.2**	57.8	69.9#	72.9#				
	18	0	100.0	100.0	100.0	100.0	1.0	1.0	3.5	2.0
	18	500	110.4	96.1	88.9	81.1	4.0	3.5	2.5	2.0
	18	1000	96.3	108.6	77.8#	81.8	1.5	1.5	3.0	1.5
	18	2000	78.4*	91.4	70.6#	66.4#	N/A			
	18	2500	53.7**	103.9	77.0#	65.7#	4.0	4.0	N/A	
	18	3000	59.7**	103.1	43.7#	65.0#	N/A		3.5	3.5
	18	+ve	74.6*	46.1	57.1#	49.0#	56.5	55.5**	74.0	71.5**
	30	0	100.0	100.0	100.0	100.0	N/A			
	30	2000	61.8**	71.2	80.6	72.2#				
	30	2500	69.9**	77.6	60.6#	70.6#				
	30	3000	78.9*	55.1	47.1#	46.1#				
18 hours	8	0	100.0	N/A	100.0	N/A	N/A			
	8	400	71.8**		95.8					
	8	600	61.5**		112.5					
	8	800	48.9**		107.5					
	18	0	100.0		100.0		3.0	3.0	N/A	
	18	100	94.5		94.9		N/A			
	18	200	87.9		80.7		4.0	4.0		
	18	400	87.9		86.9		2.5	2.0		
	18	600	63.7**		77.8#		N/A			
	18	800	69.2*		68.2#		3.5	3.5		
	18	+ve	113.2		71.0#		52.0	51.5**		

defined as relevantly different from controls by study director. * $p < 0.05$; ** $p < 0.01$ vs –ve controls. N/A = not applicable (not measured).

Table 3: Detailed chromosome aberration data from treatment of V79 cells: 4-hour treatment, 18 hour harvest (–S9)

Dose level (µg/mL)	Culture ID #	Cells Scored	Gaps		Aberration Type										Polyploid metaphases	Metaphases with aberrations (%)	
			g	ig	b	f	d	ib	if	id	ex	maE	ma	cd		incl. gaps	excl. gaps
0	6	100	0	0	0	0	0	0	0	1	0	0	0	0	13	1	1
	37	100	0	0	0	0	0	1	0	0	0	0	0	0	14	1	1
	Total	200	0	0	0	0	0	1	0	1	0	0	0	0	27	1	1
500	14	100	0	0	1	0	0	0	0	2	0	0	0	0	15	3	3
	7	100	1	0	0	0	0	1	0	3	0	0	0	0	16	5	4
	Total	200	1	0	1	0	0	1	0	5	0	0	0	0	31	4	3.5
1000	2	100	0	0	1	0	0	0	0	1	0	0	0	0	12	2	2
	42	100	0	0	0	0	0	1	0	0	0	0	0	0	10	1	1
	Total	200	0	0	1	0	0	1	0	1	0	0	0	0	22	1.5	1.5
2500	35	100	0	0	0	0	0	2	0	1	2	0	0	0	14	4	4
	12	100	0	0	1	0	0	1	0	0	3	1	0	1	15	4	4
	Total	200	0	0	1	0	0	3	0	1	5	1	0	1	29	4	4
MMC 0.1	8	100	1	1	20	2	0	19	3	6	35	1	1	0	10	60	60
	36	100	0	4	13	1	0	22	1	3	38	1	0	0	6	53	51
	Total	200	1	5	33	3	0	41	4	9	73	2	1	0	16	56.5	55.5*

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration. * $p < 0.01$ vs controls

Table 4: Detailed chromosome aberration data from treatment of V79 cells: 4-hour treatment, 18 hour harvest (+S9)

Dose level (µg/mL)	Culture ID #	Cells Scored	Gaps		Aberration Type										Polyploid metaphases	Metaphases with aberrations (%)	
			g	ig	b	f	d	ib	if	id	ex	maE	ma	cd		incl. gaps	excl. gaps
0	27	100	2	0	1	0	0	1	0	0	0	1	0	0	5	5	3
	26	100	1	0	0	0	0	1	0	0	0	0	0	0	6	2	1
	Total	200	3	0	1	0	0	2	0	0	0	1	0	0	11	3.5	2
500	17	100	0	0	0	0	0	0	1	0	0	0	0	0	7	1	1
	31	100	1	0	0	0	0	1	2	0	0	0	0	0	5	4	3
	Total	200	1	0	0	0	0	1	3	0	0	0	0	0	12	2.5	2
1000	22	100	2	0	0	0	0	0	1	0	0	0	0	0	3	3	1
	41	100	1	0	2	0	0	0	0	0	2	0	0	0	7	3	2
	Total	200	3	0	2	0	0	0	1	0	2	0	0	0	10	3	1.5
3000	19	100	0	0	3	0	0	3	0	2	0	0	0	0	11	6	6
	40	100	0	0	1	0	0	0	0	0	0	0	0	0	6	1	1
	Total	200	0	0	4	0	0	3	0	2	0	0	0	0	17	3.5	3.5
CP 2.0	5	100	8	0	53	0	0	43	5	0	21	3	5	0	5	81	78
	18	100	7	0	36	1	0	37	5	0	33	2	0	0	4	67	65
	Total	200	15	0	89	1	0	80	10	0	54	5	5	0	9	74	71.5*

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration. * $p < 0.01$ vs controls

Table 5: Detailed chromosome aberration data from treatment of V79 cells: 4-hour treatment (30-hour harvest)

Metabolic activation	Dose level (µg/mL)	Culture ID #	Cells Scored	Gaps		Aberration Type										Polyploid metaphases	Metaphases with aberrations (%)	
				g	ig	b	f	d	ib	if	id	ex	maE	ma	cd		incl. gaps	excl. gaps
–S9	0	11	100	0	1	0	0	0	0	0	1	0	0	0	0	14	2	1
		34	100	0	0	0	0	0	0	0	1	0	0	0	0	4	1	1
		Total	200	0	1	0	0	0	0	0	2	0	0	0	0	18	1.5	1
	2500	39	100	0	0	0	0	0	1	1	1	0	1	0	0	13	4	4
		38	100	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0
		Total	200	0	0	0	0	0	1	1	1	0	1	0	0	25	2	2
+S9	0	100	100	1	0	1	0	0	0	1	0	0	0	0	0	15	3	2
		100	100	3	0	0	0	0	1	0	0	0	0	0	0	23	3	1
		Total	200	4	0	1	0	0	1	1	0	0	0	0	0	38	3	1.5
	3000	100	100	0	0	0	0	0	0	1	0	0	0	0	0	21	1	1
		100	100	1	0	1	0	1	0	1	0	2	2	0	0	14	5	5
		Total	200	1	0	1	0	1	0	2	0	2	2	0	0	35	3	3

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration. * $p < 0.01$ vs controls

Table 6: Detailed chromosome aberration data from treatment of V79 cells: 18-hour treatment, 18 hour harvest (–S9)

Dose level (µg/mL)	Culture ID #	Cells Scored	Gaps		Aberration Type										Polyploid metaphases	Metaphases with aberrations (%)	
			g	ig	b	f	d	ib	if	id	ex	ma E	ma	cd		incl. gaps	excl. gaps
0	51	**100**	0	0	0	0	0	0	0	2	0	0	0	0	5	2	2
	49	**100**	0	0	1	0	0	2	3	1	0	0	0	0	6	4	4
	Total	**200**	0	0	1	0	0	2	3	3	0	0	0	0	11	3	3
200	56	**100**	0	0	2	0	0	1	0	1	0	0	0	0	7	4	4
	58	**100**	0	0	0	0	0	1	0	3	0	0	0	0	5	4	4
	Total	**200**	0	0	2	0	0	2	0	4	0	0	0	0	12	4	4
400	47	**100**	1	0	0	0	0	0	0	0	0	0	0	0	3	1	0
	52	**100**	0	0	1	1	0	1	1	0	0	0	0	0	7	4	4
	Total	**200**	1	0	1	1	0	1	1	0	0	0	0	0	10	2.5	2
800	53	**100**	0	0	2	0	0	0	0	2	0	0	0	0	11	4	4
	48	**100**	0	0	0	0	0	1	0	2	0	0	0	0	6	3	3
	Total	**200**	0	0	2	0	0	1	0	4	0	0	0	0	17	3.5	3.5
MMC 0.03	57	**100**	0	0	36	0	0	12	3	1	17	0	0	0	5	55	55
	54	**100**	1	0	31	2	0	3	3	2	23	0	0	0	4	49	48
	Total	**200**	1	0	67	2	0	15	6	3	40	0	0	0	9	52	51.5*

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration. * $p < 0.01$ vs controls

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 473 (1997) and other equivalent test guidelines.

C. CONCLUSIONS: BYI 02960 was tested up to the limit dose of 3000 µg/mL (equivalent to 10 mM) but failed to induce a clastogenic effect in Chinese Hamster V79 cells. The expected responses were obtained with the negative and positive controls either with or without S9 activation at appropriately selected time intervals. Based on these considerations, it was concluded that BYI 02960 was negative in this test system with and without metabolic activation.

Accordingly, BYI 02960 is negative in this test system for clastogenicity.

Primary review: Australia
Secondary review: US EPA & PMRA

Report:	Hall, C. (2010b) BCS-AA56716 (metabolite of BYI 02960) *In vitro* Chromosome Aberration Test with Chinese Hamster V79 Cells. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Study ID: 1351103. Ref: M-409726-01-2. MRID 48844132. Unpublished
Dates of work:	4 August – 1 October 2010. Final Report 15 December 2010.
Guidelines:	OPPTS 870.5375 (1998), EC 440/2008, B10 (2008), OECD No. 473 (1997).
GLP/QA:	OECD, US EPA, EC and JMAFF GLP-compliant Signed and dated Quality Assurance and Data Confidentiality statements were provided.

Executive Summary:

BCS-AA56716 (difluoroacetic acid) (99.6%; Lot No. BCOO 5984-4-11) was tested in the *in vitro* mammalian chromosome aberration test using Chinese Hamster V79 cells both with and without an exogenous metabolic activation system (phenobarbital/β-naphthoflavone-induced rat liver S9). In the preliminary toxicity assay, the cells were exposed to nine concentrations of the test substance ranging from 0–960 μg/mL (the limit dose) with a 4-hour treatment period. The highest dose level for the main chromosome aberration study was set based on production of cytotoxicity (or lack thereof where no treatment-related effects were observed at the limit dose) in a preliminary toxicity assay.

No test material precipitation was observed in the preliminary toxicity studies. No obvious changes in cell survival indices or mitotic indices were observed at up to the limit dose of 960 μg/mL (i.e. equivalent to approximately 10mM) after a 4-hour exposure. Chromosome aberration screening at the three top dose levels in the preliminary study (including the limit dose) did not reveal a treatment-related and biologically significant increase in aberrations. While an apparent increase in the number of aberrant gaps (4.5%) was noted at 480 μg/mL (–S9) compared with historical laboratory control data (min/max range 0–4%), the result was not considered biologically relevant noting that a similar increase was not observed at the next highest dose (which was also the limit dose).

In the main study, for Experiment 1 concentrations of 0, 240, 480 and 960 μg/mL BCS-AA56716 with 4 hours treatment and 18 hours harvest ±S9 were chosen for reading. In Experiment 2 without S9 concentration of 0, 240, 480 and 960 μg/mL BCS-AA56716 with 18 hours treatment and harvest were chosen for reading, and with S9 concentrations of 0, 240, 480 and 960 μg/mL with 4 hours treatment and 18 hours harvest were chosen for reading. No statistically significant or dose-related increase in the rate of chromosome aberrations (± gaps) was noted with test material treatment for 4 hours (±S9) or 18 hours (-S9). Positive controls elicited the expected increase in metaphases with aberrations at the different treatment time points with and without metabolic activation. While a slight decrease (~35% less than concurrent controls) in the survival index of cells was noted at the limit dose of 960 μg/mL in +S9 cultures in this experiment, the observed decrease at the limit dose was not at levels which would affect the chromosome aberration screening.

Based on the findings of this study, BCS-AA56716 was concluded to be negative for the induction of structural and numerical chromosome aberrations in cultured Chinese Hamster V79 cells with and without an exogenous metabolic activation system.

This study is classified as **reliable (acceptable/guideline)** and satisfies the guideline requirement for an i*n vitro* mammalian cytogenetics (chromosome aberrations) assay (OPPTS 870.5375; OECD 473).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BCS-AA56716 (metabolite of BYI 02960)
   Lot/Batch #: BCOO 5984-4-11
   Purity: 99.6%
   Description: Colourless liquid (as listed on Certificate of Analysis).
   CAS # Not specified
   Stability of test compound: Results from stability testing indicated that the test material was stable for at least 24 hours at 0.01 and 300 mg/mL in DMSO solvent, covering the time from formulation through to last treatment.

2. Control materials
   Solvent control: Dimethyl sulfoxide (DMSO)
   Positive, non-activation: Ethylmethane sulfonate (EMS) in nutrient medium (600 or 1000 µg/mL; equivalent to 4.8 or 8 mM respectively)
   Positive, activation: Cyclophosphamide (CP) in saline (1.4 µg/mL; equivalent to 5 µM)

3. Activation: Rat liver S9 from Male Wistar rats (phenobarbital/β-naphthoflavone-induced)
   Lot number: 210510
   Source: In-house production
   Protein content: 33.7 mg/mL
   Characterisation: The metabolic activation ability of the S9 was assayed for its metabolising capacity using 2-aminoanthracene and benzo(a)pyrene.

   S9 mix composition
   Glucose-6-phosphate: 5 mM
   NADP: 4 mM
   KCl: 33 mM
   $MgCl_2$: 8 mM
   S9: 0.75 mg/mL final concentration equivalent
   Sodium phosphate buffer (pH 7.4) 100 mM

4. Test cells

Chinese hamster V79 cells were obtained from LMP Technical University (Darmstadt, Germany). Karyotype and mycoplasma contamination of the V79 cells was checked prior to freezing and storage. On study commencement, cells were brought up from frozen stock and cultured in minimal essential media (+ Hank's salts and 10 % FBS) at 37°C prior to experimental start.

5. Culture medium (for the assays)

Minimal Essential Media (MEM), supplemented with Hank's salts, neomycin (5 µg/mL), HEPES (25 mM) and amphotericin B (2.5 µg/mL) was used during seeding and treatment of cell cultures. Where appropriate, 50 µL of S9 mix was used in S9-activated assays.

6. Test compound concentrations evaluated:

Preliminary cytotoxicity test/Experiment 1: 0, 3.8, 7.5, 15, 30, 60, 120, 240, 480, 960 µg/mL (±S9); exposure for 4-hours, with harvesting at 18 hour after dosing commencement. The top dose of 960 µg/mL was noted to be equivalent to the limit dose of 10 mM.

Main chromosome aberration test/Experiment 2:

S9-activated 4-hour exposure group:	0, 60, 120, 240, 480, 960 µg/mL
Non-activated 18-hour exposure group:	0, 3.8, 7.5, 15, 30, 60, 120, 240, 480, 960 µg/mL (repeated once as initial test did not meet acceptance criteria).

All cell cultures were set up in duplicate for each dose level, and incubated at 37°C in a 1.5% CO_2/98.5% air atmosphere.

B. STUDY DESIGN AND METHODS

Experimental start/completion:

4 August – 1 October 2010

Treatment:

The preliminary toxicity test was performed to select concentrations for the chromosome aberration assay and consisted of a cytotoxicity evaluation. The cells were exposed to solvent alone and to nine concentrations of the test substance for 4 hours in both the presence and absence of S9 activation, up to the limit dose of 960 µg/mL (equivalent to 10 mM).

Based on the preliminary results, cells were exposed to the same concentration series of test substance for 18 hours continuously in the absence of S9 activation, and in separate experiments, were administered solvent alone and five concentrations of test substance up to the limit dose of 960 µg/mL (equivalent to 10 mM). Cells were harvested at 18 hours after treatment commencement, in accordance with guideline.

In 4-hour treatment assays, cells were washed after the exposure period with "Saline G" (8 g/L NaCL, 0.4 g/L KCl, 1.1 g/L glucose, 0.192 g/L $Na_2HPO_4.2H_2O$ and 0.15 g/L KH_2PO_4), then re-suspended in culture medium (as defined in I.A.5 above) supplemented with 10 % (v/v) FBS until cell harvesting.

Cytogenetic assay: Independently performed experiments were conducted

Cell exposure time:

Non-activated:	4 and 18 h (separate series of cultures; see Section I.A.6 above)
Activated:	4 h

Spindle inhibition:

Inhibition used/concentration:	Colcemid (0.2 µg/mL)
Administration time:	2.5 hours before cell harvest

Cell harvest time after termination of treatment:	Test material	Positive control
4-hour treatment (±S9)	18 h harvest, all dose levels	18 h (EMS, 1000 µg/mL or CP, 1.4 µg/mL)
18-hour treatment (–S9)	18 h harvest, all dose levels	18 h harvest (EMS; 600 µg/mL)

Details of slide preparation:

Following the 2.5-hour colcemid treatment, cells were treated with hypotonic KCl (0.4% solution, 37°C) for 20 minutes, then fixed with a 3:1 mixture of methanol and acetic acid and stained with Giemsa.

Metaphase analysis

No. of cells examined per dose: 200 (100 per duplicate culture) were examined (except CP positive control in Experiment 1, where 50/culture were examined). Minimum 100 metaphases scored.
Mitotic index count: 1000 cells per culture

Scored for structural?	×	Yes		No
Scored for numerical?	×	Yes, polyploidy cells		No

Coded prior to analysis?	[×]	Yes	[ ]	No

Evaluation criteria:

The test was considered valid if the number of structural aberrations found in the solvent controls fell within the range of the laboratory historical controls, and the positive controls demonstrated significant increases in the number of cells with structural chromosome aberrations, which were also within the laboratory historical control range.

The test substance was considered to induce a positive response if the number of induced structural chromosome aberrations is not within the laboratory historical control range, and either a concentration-related or significant increase in the number of structural chromosome aberrations is observed.

The test substance was considered to be non-clastogenic if no such increase in structural chromosome aberrations was observed at any time interval, and/or where no significant findings were observed. Statistical and biological significance were considered in interpreting results.

Additionally, comparison of the frequency of polyploidy and endoreduplications with laboratory historical control data was also considered as part of the evaluation criteria.

Statistical analysis: Statistical analysis was conducted using the Fisher's Exact test ($p<0.05$).

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

DMSO was determined to be the solvent of choice based on known general solubility properties and relative lack of toxicity in the cell type used in the study. No stability testing of the test substance was conducted, but during the preliminary and main assays, no test substance precipitation or changes in cellular osmolarity was observed in culture medium used.

B. PRELIMINARY CYTOTOXICITY ASSAY/EXPERIMENT I.

In preliminary cytotoxicity examinations, no major changes in cell survival indices or mitotic indices were observed up to the limit dose of 960 µg/mL (Table 1). Scoring of the three top concentrations (including the limit dose) for chromosome aberrations indicated that no biologically or statistically significant changes were observed compared with concurrent and laboratory historical controls (Tables 2–3). While a slight apparent increase in the number of aberrant gaps (4.5%) was noted at 480 µg/mL (–S9) compared with historical laboratory control data (min/max range 0–4%), the result was not considered biologically

relevant noting that a similar increase was not observed at the next highest dose (which was also the limit dose). On the basis of these findings, and noting that the preliminary assay was conducted in a manner equivalent to the main assay, the preliminary cytotoxicity experiment was designated Experiment I and the results used as part of the evaluation of the test substance for clastogenic effects.

Table 1: Survival and Mitotic indices from preliminary cytotoxicity study/Experiment I with BCS-AA56716.

Treatment time	Dose level (µg/mL)	Mitotic Index (%)		Survival Index (%)	
		–S9	+S9	–S9	+S9
4 hours	0	100.0	100.0	100.0	100.0
	3.8	--	--	--	--
	7.5	--	--	--	--
	15	--	--	--	--
	30	--	--	--	--
	60	104.2	106.4	84.9	122.7
	120	90.5	111.3	120.0	95.2
	240	91.7	106.9	111.8	121.3
	480	106.0	112.8	113.7	99.7
	960	90.5	103.9	115.5	119.8
	+ve control	94.0	71.4	--	--

-- = not recorded (sufficient data from other dose levels)

Table 2: Detailed chromosome aberration data from treatment of V79 cells with BCS-AA56716: 4-hour treatment, 18 hour harvest (–S9)

Dose level (µg/mL)	Culture ID #	Cells Scored	Gaps		Aberration Type										Metaphases with aberrations (%)		
			g	ig	b	f	d	ex	ib	if	id	cx	ma	cd	incl. gaps	excl. gaps	with exchanges
0	1	**100**	0	0	2	0	0	1	0	0	0	0	0	0			
	2	**100**	0	0	1	0	0	2	0	0	0	0	0	0			
	Total	**200**	0	0	3	0	0	3	0	0	0	0	0	0	3.0	3.0	1.5
240	1	**100**	1	0	3	0	0	3	0	1	0	0	0	0			
	2	**100**	0	0	7	2	0	1	1	0	0	0	0	0			
	Total	**200**	1	0	10	2	0	4	1	1	0	0	0	0	3.8	3.5	0.8
480	1	**100**	1	0	7	0	0	4	3	1	0	0	0	0			
	2	**100**	2	0	2	1	0	3	1	0	0	0	0	0			
	Total	**200**	3	0	9	1	0	7	4	1	0	0	0	0	4.8	4.5	1.5
960	1	**100**	0	0	4	1	0	0	0	0	0	0	0	0			
	2	**100**	1	0	1	0	0	0	0	0	0	0	0	0			
	Total	**200**	1	0	5	1	0	0	0	0	0	0	0	0	2.5	2.0	0.0
EMS 1000	1	**100**	3	0	9	1	0	14	0	0	0	0	0	0			
	2	**100**	1	0	11	0	0	14	1	0	0	0	0	0			
	Total	**200**	4	0	20	1	0	28	1	0	0	0	0	0	39.0	37.0	22.0

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration.

Table 3: Detailed chromosome aberration data from treatment of V79 cells with BCS-AA56716: 4-hour treatment, 18 hour harvest (+S9)

Dose level (µg/mL)	Culture ID #	Cells Scored	Gaps		Aberration Type										Metaphases with aberrations (%)		
			g	ig	b	f	d	ex	ib	if	id	cx	ma	cd	incl. gaps	excl. gaps	with exchanges
0	1	**100**	0	0	1	1	0	0	0	0	0	0	0	0			
	2	**100**	0	0	5	0	0	0	0	0	0	0	0	0			
	Total	**200**	0	0	6	1	0	0	0	0	0	0	0	0	3.5	3.5	0.0
240	1	**100**	1	0	1	0	0	0	0	0	0	0	0	0			
	2	**100**	0	0	2	0	0	0	0	0	0	0	0	0			
	Total	**200**	1	0	3	0	0	0	0	0	0	0	0	0	1.5	1.0	0.0
480	1	**100**	0	0	0	0	0	0	0	0	0	0	0	0			
	2	**100**	0	0	2	0	0	0	0	0	0	0	0	0			
	Total	**200**	0	0	2	0	0	0	0	0	0	0	0	0	1.0	1.0	0.0
960	1	**100**	0	0	2	1	0	0	1	0	0	0	0	0			
	2	**100**	0	0	4	0	0	0	0	0	0	0	0	0			
	Total	**200**	0	0	6	1	0	0	1	0	0	0	0	0	3.5	3.5	0.0
CP 1.4	1	**100**	0	0	5	0	0	4	1	0	0	0	0	0			
	2	**100**	1	0	5	0	0	6	0	0	0	0	0	0			
	Total	**200**	1	0	10	0	0	10	1	0	0	0	0	0	9.0	8.5	4.5

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration.

C. MAIN CHROMOSOME ABERRATION ASSAY/EXPERIMENT 2

Summarized results from the chromosome aberration assays are presented in Tables 4–6 below and are as follows:

Without metabolic activation (–S9): The survival and mitotic indices for cells treated with the test substance were unchanged compared with concurrent negative controls at up to the limit dose of 960 µg/mL.

With metabolic activation (+S9): The mitotic index for cells treated with the test substance was unchanged compared with negative control at up to the limit dose. A slight decrease in the survival index for cells was noted at the limit dose of 960 µg/mL, with a ~35% decrease in cells observed at this dose. However, the observed decrease in cell count at the limit dose was not at levels which would affect the chromosome aberration screening.

Based on the survival and mitotic index data from the main study, at least three relevant dose levels from cultures with and without metabolic activation were selected for chromosome aberration screening. Results from chromosome aberration screening are shown in Tables 4–6 below. In all cases, no statistically significant or dose-related increase in the rate of chromosome aberrations (± gaps) was noted with test material treatment. Positive controls elicited the expected increase in metaphases with aberrations at the different treatment time points with and without metabolic activation.

No polyploidy metaphases were observed in either experiment.

Table 4: Survival and Mitotic indices from main chromosome aberration assay (Experiment II) with BCS-AA56716.

Treatment time	Dose level (µg/mL)	Mitotic Index (%)	Survival Index (%)
4 hours (+S9)	**0**	100.0	100.0
	60	121.4	101.3
	120	80.9	90.3
	240	102.7	93.9
	480	10.5	85.5
	960	87.7	64.9
	+ve control	98.2	--
18 hours (–S9)	**0**	100.0	100.0
	60	117.6	89.6
	120	96.9	103.9
	240	107.5	87.3
	480	106.3	84.0
	960	108.2	107.1
	+ve control	89.9	--

-- = not recorded (suficeint data from other sources).

Table 5: Detailed chromosome aberration data from treatment of V79 cells (Experiment II): 4-hour treatment, 18 hour harvest (+S9)

Dose level (µg/mL)	Culture ID #	Cells Scored	Gaps		Aberration Type										Metaphases with aberrations (%)		
			g	ig	b	f	d	ex	ib	if	id	cx	ma	cd	incl. gaps	excl. gaps	with exchanges
0	1	**100**	0	0	2	0	0	1	0	0	0	0	0	0			
	2	**100**	1	0	1	0	0	0	0	0	0	0	0	0			
	Total	**200**	1	0	3	0	0	1	0	0	0	0	0	0	2.5	2.0	0.5
240	1	**100**	0	0	2	0	0	0	0	0	0	0	0	0			
	2	**100**	0	0	2	0	0	1	0	0	0	0	0	0			
	Total	**200**	0	0	4	0	0	1	0	0	0	0	0	0	2.0	2.0	0.5
480	1	**100**	0	0	0	0	0	1	0	1	0	0	0	0			
	2	**100**	0	0	1	0	0	1	0	0	0	0	0	0			
	Total	**200**	0	0	1	0	0	2	0	1	0	0	0	0	2.0	2.0	1.0
960	1	**100**	1	0	1	0	0	0	0	0	0	0	0	0			
	2	**100**	0	0	1	0	0	0	0	0	0	0	0	0			
	Total	**200**	1	0	2	0	0	0	0	0	0	0	0	0	1.5	1.0	0.0
CP 1.4	1	**100**	7	0	3	1	0	2	0	4	0	0	1	0			
	2	**100**	3	0	9	0	0	6	1	0	0	0	0	0			
	Total	**200**	10	0	12	1	0	8	1	4	0	0	1	0	15.0	12.0	4.0

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration.

Table 6: Detailed chromosome aberration data from treatment of V79 cells (Experiment II): 18-hour treatment, 18 hour harvest (–S9)

Dose level (µg/mL)	Culture ID #	Cells Scored	Gaps		Aberration Type										Metaphases with aberrations (%)		
			g	ig	b	f	d	ex	ib	if	id	cx	ma	cd	incl. gaps	excl. gaps	with exchanges
0	1	**100**	0	0	1	0	0	0	1	0	0	0	0	0			
	2	**100**	1	0	0	1	0	0	1	0	0	0	0	0			
	Total	**200**	1	0	1	1	0	0	2	0	0	0	0	0	2.5	2.0	0.0
240	1	**100**	1	0	1	0	0	0	0	0	0	0	0	0			
	2	**100**	0	0	0	0	0	0	0	0	0	0	0	0			
	Total	**200**	1	0	1	0	0	0	0	0	0	0	0	0	1.0	0.5	0.0
480	1	**100**	0	0	0	0	0	0	0	0	0	0	0	0			
	2	**100**	0	0	0	0	0	0	0	0	0	0	0	0			
	Total	**200**	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0
960	1	**100**	0	0	3	0	0	0	0	0	0	0	0	0			
	2	**100**	0	0	0	0	0	0	0	0	0	0	0	0			
	Total	**200**	0	0	3	0	0	0	0	0	0	0	0	0	1.5	1.5	0.0
EMS 600	1	**100**	1	0	11	1	0	6	3	0	0	0	1	0			
	2	**100**	0	0	12	1	0	6	0	0	0	0	0	0			
	Total	**200**	1	0	23	2	0	12	3	0	0	0	1	0	17.5	17.0	6.0

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration.

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 473 (1997) and other equivalent test guidelines.

C. CONCLUSIONS: BCS-AA56716 was tested up to the limit dose of 960 μg/mL (equivalent to 10 mM) but failed to induce a clastogenic effect in Chinese Hamster V79 cells. The expected responses were obtained with the negative and positive controls either with or without S9 activation at appropriately selected time intervals. Based on these considerations, it was, therefore, concluded that BCS-AA56716 was negative in this test system with and without metabolic activation.

Accordingly, BCS-AA56716 is negative in this test system for clastogenicity.

Primary review: Australia
Secondary review: US EPA & PMRA

B.6.8.3/15 *In vitro* Mammalian Chromosome Aberration Test

Report:	Hall, C. (2011a) BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960) *In vitro* Chromosome Aberration Test in Chinese Hamster V79 Cells. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Study ID: 1399703. Ref: 420108-01-2. MRID 48844133. Unpublished.
Dates of work:	16 March – 15 April 2011. Final Report 7 October 2011.
Guidelines:	OPPTS 870.5375 (1998), EC 440/2008, B10 (2008), OECD No. 473 (1997).
GLP/QA:	OECD, US EPA, EC and JMAFF GLP-compliant Signed and dated Quality Assurance and Data Confidentiality statements were provided.

Executive Summary:

BYI 02960-difluoroethyl-amino-furanone (98.5%; Lot No. NLL 8671-6-1) was tested in the *in vitro* mammalian chromosome aberration test using Chinese Hamster V79 cells both with and without an exogenous metabolic activation system (phenobarbital/β-naphthoflavone-induced rat liver S9). In the preliminary toxicity assay, the cells were exposed to nine concentrations of the test substance ranging from 0–1636 µg/mL (the limit dose) with a 4-hour treatment period. Based on the cytotoxicity results and subsequent chromosome aberration screening findings on all cultures without metabolic activation and the three top dose levels (including the limit dose) with metabolic activation, a second confirmatory assay was not required.

No test material precipitation was observed in the studies. No obvious changes in cell survival indices or mitotic indices were observed at up to the limit dose of 1636 µg/mL (i.e. approximately 10 mM) after a 4-hour exposure with or without metabolic activation. In both series of cultures with and without activation, an elevated frequency of polyploid and endomitotic cells was noted at a number of dose levels compared with concurrent controls. Additionally, the frequency of polyploid cells was above the maximum range observed in laboratory historical controls.

Chromosome aberration screening of treated cells without metabolic activation revealed a treatment-related increase in aberrant cells across a majority of dose levels tested, including the limit dose of 1636 µg/mL. The frequency of aberrations was outside both concurrent and laboratory historical controls.

Chromosome aberration screening of treated cells with metabolic activation did not produce a treatment-related increase in chromosome aberrations at up to and including the limit dose.

Based on the findings of this study, BYI 02960-difluoroethyl-amino-furanone was concluded to be positive for the induction of structural and numerical chromosome aberrations in cultured Chinese Hamster V79 cells without an exogenous metabolic

activation system. However, BYI 02960-difluoroethyl-amino-furanone was negative for the induction of structural and numerical chromosome aberrations with an exogenous metabolic activation system.

This study is classified as **reliable (acceptable/guideline)** and satisfies the guideline requirement for an i*n vitro* mammalian cytogenetics (chromosome aberration) assay (OPPTS 870.5375; OECD 473).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960-difluoroethyl-amino-furanone (metabolite of BYI 02960)

Lot/Batch #:	NLL 8671-6-1
Purity:	98.5%
Description:	Not specified
CAS #	Not specified
Stability of test compound:	No pre-experiment testing was conducted. Osmolarity and pH values were recorded in solvent control and the top dose of 1636 µg/mL in the preliminary experiment, which indicated no treatment-related differences in these parameters, suggesting test compound stability under the conditions of the study.

2. Control materials

Solvent control:	Distilled water
Positive, non-activation:	Ethylmethane sulfonate (EMS) in nutrient medium (1000 µg/mL; equivalent to 8 mM)
Positive, activation:	Cyclophosphamide (CP) in saline (1.4 µg/mL; equivalent to 5 µM)

3. Activation: Rat liver S9 from Male Wistar rats (phenobarbital/β-naphthoflavone-induced)

Lot number:	291010
Source:	In-house production
Protein content:	35.2 mg/mL
Characterisation:	The metabolic activation ability of the S9 was assayed for its metabolising capacity using 2-aminoanthracene and benzo(a)pyrene.

S9 mix composition

Glucose-6-phosphate:	5 mM
NADP:	4 mM
KCl:	33 mM
$MgCl_2$:	8 mM
S9:	0.75 mg/mL final concentration equivalent
Sodium phosphate buffer (pH 7.4)	100 mM

4. Test cells

Chinese hamster V79 cells were obtained from LMP Technical University (Darmstadt, Germany). Karyotype and mycoplasma contamination of the V79 cells was checked prior

to freezing and storage. On study commencement, cells were brought up from frozen stock and cultured in minimal essential media (+ Hank's salts and 10 % FBS) at 37°C prior to experimental start.

5. Culture medium (for the assays)

Minimal Essential Media (MEM), supplemented with Hank's salts, neomycin (5 μg/mL), HEPES (25 mM) and amphotericin B (2.5 μg/mL) was used during seeding and treatment of cell cultures. Where appropriate, 50 μL of S9 mix was used in S9-activated assays.

6. Test compound concentrations evaluated:

In the preliminary cytotoxicity test (Experiment 1) the following dosing series was used: 0, 6.4, 12.8, 25.6, 51.1, 102.3, 204.5, 409, 818, 1636 μg/mL (±S9); exposure for 4-hours, with harvesting at 18 hour after dosing commencement. The top dose of 1636 μg/mL was noted to be equivalent to the limit dose of 10 mM.

All cell cultures were set up in duplicate for each dose level, and incubated at 37°C in a 1.5% CO_2/98.5% air atmosphere.

B. STUDY DESIGN AND METHODS

Experimental start/completion:

16 March – 15 April 2011

Treatment:

A preliminary toxicity test was performed to select concentrations for the chromosome aberration assay and consisted of a cytotoxicity evaluation. The cells were exposed to solvent alone and to nine concentrations of the test substance for 4 hours in both the presence and absence of S9 activation, up to the limit dose of 1636 μg/mL (equivalent to 10 mM).

Cells were washed after the exposure period with "Saline G" (8 g/L NaCL, 0.4 g/L KCl, 1.1 g/L glucose, 0.192 g/L $Na_2HPO_4.2H_2O$ and 0.15 g/L KH_2PO_4; pH 7.2), then re-suspended in culture medium (as defined in I.A.5 above) supplemented with 10 % (v/v) FBS until cell harvesting. Cells were harvested at 18 hours after treatment commencement, in accordance with guideline.

Based on the validity of the preliminary study findings as measured against evaluation criteria, including cytotoxicity evaluation and chromosome aberration screening, this preliminary experiment was also designated as the main experiment, in accordance with test guideline principles.

Cytogenetic assay: Independently performed experiments were conducted

Spindle inhibition:

Inhibition used/concentration:	Colcemid (0.2 μg/mL)

Administration time: 2.5 hours before cell harvest

Cell harvest time after termination of treatment:	Test material	Positive control
4-hour treatment (±S9)	18 h harvest, all dose levels	18 h (EMS, 1000 µg/mL or CP, 1.4 µg/mL)

Details of slide preparation:

Following the 2.5-hour colcemid treatment, cells were treated with hypotonic KCl (0.4% solution, 37°C) for 20 minutes, then fixed with a 3:1 mixture of methanol and acetic acid and stained with Giemsa.

Metaphase analysis

No. of cells examined per dose: 200 (100 per duplicate culture) were examined (except EMS positive control, where 50/culture were examined). Minimum 100 metaphases scored.

Mitotic index count: 1000 cells per culture

Scored for structural?	×	Yes		No
Scored for numerical?	×	Yes, polyploidy cells		No
Coded prior to analysis?	×	Yes		No

Evaluation criteria:

The test was considered valid if the number of structural aberrations found in the negative solvent controls fell within the range of the laboratory historical controls, and the positive controls demonstrated significant increases in the number of cells with structural chromosome aberrations, which were also within the laboratory historical control range.

The test substance was considered to induce a positive response if the number of induced structural chromosome aberrations is not within the laboratory historical control range, and either a concentration-related or significant increase in the number of structural chromosome aberrations is observed.

The test substance was considered to be non-clastogenic if no such increase in structural chromosome aberrations was observed at any time interval, and/or where no significant findings were observed. Statistical and biological significance were considered in interpreting results.

Additionally, comparison of the frequency of polyploidy and endoreduplications with laboratory historical control data was also considered as part of the evaluation criteria.

Statistical analysis: Statistical analysis was conducted using the Fisher's Exact test ($p<0.05$).

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Distilled water was determined to be the solvent of choice based on known general solubility properties and relative lack of toxicity in the cell type used in the study. No stability testing of the test substance was conducted, but during the preliminary assays, no test substance precipitation or changes in cellular osmolarity was observed in culture medium used.

B. PRELIMINARY CYTOTOXICITY ASSAY/EXPERIMENT I.

In preliminary cytotoxicity examinations, no major changes in cell survival indices or mitotic indices were observed up to the limit dose of 1636 µg/mL (Table 1). On the basis of these findings, chromosome aberration screening was conducted across all dose levels without S9 metabolic activation, and at the top three dose levels (including the limit dose) with S9 metabolic activation to evaluate the clastogenic potential of the test substance.

Table 1: General cell indices and properties from cytotoxicity study with BYI 02960-difluoroethyl-amino-furanone.

Metabolic activation	Dose level (µg/mL)	Mitotic Index (%)	Survival Index (%)	Polyploid cells (%)	Endomitotic cells (%)
	0	100.0	100.0	4.4	0.5
	6.4	121.7	88.6	4.3	0.6
	12.8	112.1	97.2	5.2	0.3
	25.6	116.5	95.0	5.0	0.6
	51.1	99.6	105.7	4.0	0.3
–S9	**102.3**	92.6	96.6	6.4	1.0
	204.5	117.3	89.3	9.3	1.7
	409.0	86.0	102.7	8.9	0.5
	818.0	87.1	91.1	8.1	0.4
	1636.0	88.6	85.8	6.6	0.9
	+ve control	94.9	--	3.8	0.0
	0	100.0	100.0	4.5	0.3
	6.4	--	--	--	--
	12.8	--	--	--	--
	25.6	--	--	--	--
	51.1	--	--	--	--
+S9	**102.3**	102.7	77.4	--	--
	204.5	96.0	87.7	--	--
	409.0	106.7	91.0	2.7	0.0
	818.0	112.5	88.0	4.5	0.4
	1636.0	97.9	103.6	3.5	0.1
	+ve control	65.0	--	3.9	0.0

-- = not recorded (sufficient data from other dose levels/sources)

In cultures treated with the test substance without metabolic activation, an apparent treatment-related increase in polyploidy was noted (up to 9.3 %) compared with concurrent negative

controls (4.4 %); results were also typically outside the range identified in laboratory historical controls (1.2–4.2 % for aqueous solutions, Table 2). While no laboratory historical control data were available for the frequency of endomitotic cells, an increase in the endomitotic cell frequency (up to 1.7 %) was noted in a subset of dose levels compared with concurrent solvent controls (0.5 %).

Table 2: Percentage of aberrant and polyploidy cells in Chinese hamster V79 cell cultures (January 2008 to December 2009)

	Aberrant cells (%)				Polyploid cells (%)			
	No. of studies	**Range**	**Mean**	**Standard dev.**	**No. of studies**	**Range**	**Mean**	**Standard dev.**
Without S9 mix: preparation interval 18 hrs, treatment 4 hrs								
Aqueous solution[1]	21	0.0 - 3.5	1.6	1.0	10	1.2 - 4.2	2.5	0.8
Organic solvent[2]	51	0.0 - 4.0	1.8	1.0	30	1.2 – 4.6	2.7	0.9
Solvent control (total)	72	0.0 - 4.0	1.8	1.0	40	1.2 – 4.6	2.8	0.9
Positive control: EMS 400-1200 µg/mL	72	8.0 - 54.0	19.1	11.3				
With S9 mix: preparation interval 18 hrs, treatment 4 hrs								
Aqueous solution[1]	31	0.0 - 3.5	1.8	0.9	15	2.0 - 4.7	2.9	0.7
Organic solvent[2]	68	0.0 - 4.0	1.7	1.1	41	0.7 - 5.6	2.9	1.1
Solvent control (total)	99	0.0 - 4.0	1.8	1.0	56	0.7 - 5.6	2.9	1.0
Positive control: CPA 1.0-2.0 µg/mL	101	8.0 - 46.0	13.2	5.4				

[1]Aqueous solvents; deionised water (10% v/v), 0.9 (w/v) saline (10% v/v) and culture MEM

[2]Organic solvents; dimethyl sulfoxide, acetone, ethanol and tetrahydrofurane (0.5% v/v)

In cultures treated with the test substance with metabolic activation, no treatment-related changes in polyploidy or frequency of endomitotic cells were observed.

Chromosome aberration screening of treated cells without metabolic activation indicated a treatment-related increase in the frequency of aberrant cells (with and without gaps). The observed increase in aberrant cells was outside the concurrent and laboratory historical control values, but below the concurrent positive control (Tables 3–4). In treated cells with metabolic activation, no treatment-related increase in chromosome aberration frequency was observed at up to the limit dose of 1636 µg/mL.

Table 3: Detailed chromosome aberration data from treatment of V79 cells (Experiment I): 4-hour treatment, 18 hour harvest (–S9)

Dose level (µg/mL)	Gaps		Aberration Type										Metaphases with aberrations (%)		
	g	ig	b	f	d	ex	ib	if	id	cx	ma	cd	incl. gaps	excl. gaps	with exchanges
0	0	0	1	0	0	1	0	0	0	0	0	0	1.0	1.0	0.5
6.4	0	0	3	0	0	5	0	0	0	0	1	0	4.0	4.0	2.0
12.8	0	0	5	0	0	4	0	0	0	0	1	0	4.5	4.5	2.0
25.6	3	0	9	3	0	11	0	0	0	1	1	1	9.0	8.5	5.0
51.1	1	0	11	2	0	4	1	1	0	1	1	0	8.5	8.0	2.0
102.3	0	0	3	1	0	6	1	1	0	0	0	0	5.5	5.5	3.0
204.5	1	0	3	1	0	5	1	0	0	0	0	0	4.5	4.0	2.5
409.0	1	0	4	0	0	11	0	0	0	0	0	0	5.0	4.5	3.5
818.0	1	0	11	0	0	9	1	0	0	0	0	0	7.5	7.5	4.5
1636.0	1	0	5	0	0	9	2	0	0	0	2	0	8.0	7.5	4.0
EMS (1000)	0	0	5	1	0	48	2	0	0	1	0	0	39.0	39.0	36.0

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration. 200 cells scored per dose level, except positive control EMS, where 100 cells scored only due to strong clastogenic effects.

Table 4: Detailed chromosome aberration data from treatment of V79 cells (Experiment I): 4-hour treatment, 18 hour harvest (+S9)

Dose level (µg/mL)	Culture ID #	Cells Scored	Gaps		Aberration Type										Metaphases with aberrations (%)		
			g	ig	b	f	d	ex	ib	if	id	cx	ma	cd	incl. gaps	excl. gaps	with exchanges
0	1	**100**	0	0	2	0	0	1	1	0	0	0	0	0			
	2	**100**	0	0	5	0	0	0	0	0	0	0	0	0			
	Total	**200**	0	0	7	0	0	1	1	0	0	0	0	0	3.0	3.0	0.5
409	1	**100**	0	0	0	0	0	0	0	0	0	0	0	0			
	2	**100**	0	0	1	0	0	0	0	0	0	0	0	0			
	Total	**200**	0	0	1	0	0	0	0	0	0	0	0	0	0.5	0.5	0.0
818	1	**100**	1	0	1	1	0	0	0	0	0	0	0	0			
	2	**100**	0	0	0	1	0	0	0	0	0	0	0	0			
	Total	**200**	1	0	1	2	0	0	0	0	0	0	0	0	1.5	1.0	0.0
1636	1	**100**	0	0	2	0	0	1	0	0	0	0	0	0			
	2	**100**	0	0	1	2	0	2	0	0	0	0	0	0			
	Total	**200**	0	0	3	2	0	3	0	0	0	0	0	0	2.0	2.0	1.0
CP 1.4	1	**100**	0	0	8	0	0	4	1	1	0	0	0	0			
	2	**100**	1	0	8	2	0	1	1	0	0	0	1	0			
	Total	**200**	1	0	16	2	0	5	2	1	0	0	1	0	12.5	12.0	2.5

g = gap; ig = isogap; b = break; ib = isobreak; f = fragment; if = isofragment; d = deletion; id = isodeletion; ex = exchange; ma = multiple aberrations; maE = multiple aberrations with exchange; cd = chromosome disintegration. 200 cells scored per dose level.

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 473 (1997) and other equivalent test guidelines.

C. CONCLUSIONS

BYI 02960-difluoroethyl-amino-furanone was tested up to the limit dose of 1636 µg/mL (equivalent to 10 mM). In treated cells without metabolic activation, an increased frequency of chromosome aberrations was noted across the majority of dose levels tested, including up to the limit dose of 1636 µg/mL, indicating a treatment-related clastogenic effect. In treated cells with metabolic activation, no clastogenic effect was observed with treatment up to the limit dose. The expected responses were obtained with the negative and positive controls either with or without S9 activation at appropriately selected time intervals. Based on these considerations, it was, therefore, concluded that BYI 02960-difluoroethyl-amino-furanone was positive for clastogenicity in this test system in a well-conducted study.

Accordingly, BYI 02960-difluoroethyl-amino-furanone is positive in this test system for clastogenicity in the absence of metabolic activation only (i.e. negative for clastogenciity in the presence of metabolic activation).

Primary reviewer: Australia
Secondary reviewer: US EPA & PMRA

In Vivo Genotoxicity (somatic cells) – Bone marrow or micronucleus

Report:	Herbold, B (2009b). BYI 02960: Micronucleus Test on the Male Mouse. Bayer Schering Pharma AG, Wuppertal, Germany. Study Report: T0080066; Report No: AT05350. Ref: M-353785-01-2. MRID 48844134. Unpublised.
Dates of work:	31 March – 2 June 2009. Final Report 9 July 2009.
Guidelines:	OPPTS 870. 5395 (1998); OECD 474 (1997); EEC 2000/32/EC Method B12 (2000).
GLP/QA:	OECD, US EPA, Germany, EC and JMAFF GLP compliant. Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary:

BYI 02960 technical (96.2%; Lot No. 2009-000239) was evaluated in the mouse micronucleus assay for its ability to induce micronuclei in bone marrow polychromatic erythrocytes (PCEs) in male Crl:NMRI BR mice (5/dose level). Doses were 0, 10, 20 and 40 mg/kg by two intra-peritoneal injections 24 hours apart, based on the range finding results indicating 40 mg/kg bw was the maximum tolerated dose. Concurrent control groups were administered corn oil (the vehicle (negative) control), or 20 mg/kg of cyclophosphamide (positive control). All animals were sacrificed 24 hours after the second i.p. injection. Bone marrow smears were prepared immediately after the sacrifices. In-life observations included clinical signs. Two thousand PCEs per animal were evaluated for micronuclei.

In the main study, there were no deaths, though treatment-related toxicity was observed, including apathy, roughened fur, weight loss, sternal recumbency, spasm, periodic stretching of the body and difficulty breathing. No evidence of a cytotoxic effect on the bone marrow cells was observed, while the positive and vehicle controls induced the expected response. No statistically significant increases in micronucleated PCE frequency were observed in any evaluated test substance-treated group of animals.

Under the conditions of this study, BYI 02960 was neither clastogenic nor aneugenic in the mouse bone marrow micronucleus assay.

This study is classified as **reliable** (**acceptable/guideline)** and satisfies the guideline requirement (OPPTS 870.5395; OECD 474) for *in vivo* cytogenetic -erythrocyte micronucleus assay in mice mutagenicity data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   Lot/Batch #: 2009-000239
   Purity: 96.2%
   Description: Fine light brown powder
   CAS #: 951659-40-8
   Stability of test compound: Stability analyses indicated that the test material was stable at 0.1 and 10 mg/mL concentrations for at least 4 hours
2. Control materials
   Negative: Corn oil
   Positive: Cyclophosphamide (CP) at 20 mg/kg bw
3. Test animals
   Species: Mouse (male only)
   Strain: Crl:NMRI BR
   Age at dosing: Approximately 6–12 weeks
   Weight at dosing: 37–43 g
   Source: Charles River Deutschland, Sulzfeld, Germany
   Acclimation period: At least 5 days
   Diet: Fixed-formula feed 3883, Provimi Kilba SA, Kaiseraugst. .
   Water: Tap water, *ad libitum*
   Housing: Individually in Type II cages, with Lignocel BK 8/15 soft wood granules for bedding.
4. Environmental conditions
   Temperature: 22 ± 1.5°C
   Humidity: 40-70%
   Air changes: Approx. 10 changes/hour
   Photoperiod: Alternating 12-hour light (6am–6pm) and dark cycles
5. Number of animals per dose
   Range-finding test: 3/sex/dose
   Micronucleus assay: 5 males/dose (additional replacement animal group for top dose)
6 Test compound concentrations used
   Range-finding test: 10, 40, 100 or 1000 mg/kg bw BYI 02960 by i.p. injection
   Micronucleus assay: 0, 10, 20 or 40 mg/kg bw BYI 02960 by i.p. injection.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

31 March – 2 June 2009

2. Range-finding test

Dosing concentrations used were determined by the range finding results from an experiment using groups of 3 animals/sex/dose. The highest dose used for the rangefinder for the *in vivo* micronucleus study was 1000 mg/kg body weight administered by two separate i.p injections separated by 24 hours. The animals were observed for clinical signs of toxicity and mortality immediately after dosing and until at least 24 hours after the second injection.

3. Micronucleus assay

a. Treatment and sampling times:

Test compound and vehicle control

Dosing:	☐	Once	☒	twice (24 hrs apart)	☐	Other			
Sampling (after last dose):	☐	6 hr	☐	12 hr	☒	24 hr	☐	48 hr	☐ 72 hr

Positive control

Dosing:	☒	Once	☐	twice (24 hrs apart)	☐	Other			
Sampling (after last dose):	☐	6 hr	☐	12 hr	☒	24 hr	☐	48 hr	☐ 72 hr

b. Tissues and cells examined**:**

Bone marrow	
No. of polychromatic erythrocytes (PCE) examined per animal:	2000
No. of total erythrocytes examined per animal:	2000

4. Details of slide preparation**:** Immediately after sacrifice at 24 hours after the final injection, bone marrow was collected from at least one femur per animal, aspirated and mixed with fetal calf serum (FCS), centrifuged and placed onto slides. Bone marrow smears were stained with an Ames Hema-Tek Slide Stainer (Miles Company). Prepared slides were coded prior to evaluation and examined for the incidence of micronucleated polychromatic erythrocytes (MPCE) and the ratio of polychromatic erythrocytes (PCE) to total normochromatic erythrocytes (NCE). 2000 PCEs were counted per animal.

5. Evaluation criteria:

The following evaluation criteria were applied:

- The assay was considered valid and acceptable if the figure of negative and positive controls were within the expected range, from historical/published data.
- A positive response was noted as a relevant and significant increase in the number of polychromatic erythrocytes showing micronuclei in comparison to the negative control.
- A negative response was noted where no relevant or significant increase in the rate of micronucleated polychromatic erythrocytes was observed, or where a significant increase in the rate which was within historical negative controls.
- An equivocal response was noted if there was an increase in micronucleated polychromatic erythrocytes above historical negative controls, but the effect was not significant and the result of the negative control was not closely related to the data from the treatment group in question. An equivocal response was also noted where a described response was considered implausible. In both cases, a second test would normally be performed.

6. Statistical methods:

The numbers of MPCE and NCE from groups where the highest mean values occurred, and the positive control data, were checked by Wilcoxon's non-parametric rank sum test, with statistical significant at 5%. The rate of NCEs containing micronuclei was then also measured if the MPCE rate was relevantly increased through a one-sided χ^2 test at 5% statistical significance. No statistics were conducted on body weights or clinical signs.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Data from the analysis of dosing formulations indicated that the test material was stable at room temperature for approximately 4-5 hours at concentrations of 0.1 and 10 mg/mL.

B. RANGE-FINDING TEST

In the range-finding test, full mortality was observed in animals ≥ 100 mg/kg bw. Clinical symptoms related to treatment were recorded at all treatment levels (≥ 10 mg/kg bw). These included apathy, sternal recumbency, spasm, periodic stretching of body and difficulty breathing. Symptoms in animals surviving to sacrifice were noted for up to at least 24 hours after the second i.p. injection of test material. The study director indicated that based on the distribution of clinical symptoms and mortalities, 40 mg/kg bw was considered as the MTD under the conditions of the study. No substantial differences were reported between sexes and hence males only were used in the micronucleus assay.

B. MICRONUCLEUS ASSAY

1. Toxicity

No mortality occurred during the study. Treatment-related clinical symptoms were recorded in all groups administered the test material. These included apathy, roughened fur, weight loss, sternal recumbency, spasm, periodic stretching of the body and difficulty breathing.

2. PCE ratio

A slight decrease in the NCE/PCE ratio was noted across treatment groups, though no dose response pattern or statistical significance was observed.

3. Micronucleated polychromatic erythrocytes (MPCEs)

No statistically significant increases in micronucleated PCE frequency were observed in any evaluated treatment group. By contrast, the positive control (CP, 20 mg/kg) induced a significant ($p<0.01$) increase in MPCEs/2000 PCEs. Summarised results from the micronucleus assay are presented in Tables 1 and 2.

Table 1. *In vivo* micronucleus assay in male and female mice treated with BYI 02960 : PCE frequency

Time (h)	Sex	Number of animals	Mean NCE per 2000 PCE ratio				
			Vehicle control 0 mg/kg bw	Low dose 10 mg/kg bw	Mid dose 20 mg/kg bw	High dose 40 mg/kg bw	Positive control CP 20 mg/kg bw
24	male	5	3800 ± 1176	3134 ± 1213	3139 ± 1543	3008 ± 878	3308 ± 1364

CP = cyclophosphamide
Vehicle control = Corn oil

Data were derived from the study report, Tables 1–6, pp. 50–55

Table 2. *In vivo* micronucleus: assay in male and female mice treated with BYI 02960 MPCE frequency

Time (h)	Sex	Number of animals	Mean micronucleated PCE/2000 PCE				
			Vehicle control 0 mg/kg bw	Low dose 500 mg/kg bw	Mid dose 1000 mg/kg bw	High dose 2000 mg/kg bw	Positive control CP 40 mg/kg bw
24	male	5	3.4 ± 2.7	1.4 ± 1.5	3.0 ± 1.2	3.8 ± 2.3	20.6 ± 8.6*

[a] Statistically significant difference from control at $p < 0.01$ by non-parametric Wilcoxon ranking test.
CP = cyclophosphamide
Vehicle control = corn oil Data derived from the study report, Tables 1–6, pp. 50–55

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 474 (1997) and other equivalent guidelines.

C. CONCLUSIONS:

BYI 02960 did not induce an increase in micronuclei in bone marrow cells of mice at doses up to and including the maximum tolerated dose of 40 mg/kg bw (as defined by the study director from a range-finding study). The sensitivity of the test system to detect a biologically relevant and significant ($p<0.01$) increase in MPCEs was adequately demonstrated by the results obtained with the positive control, CP.

Accordingly, BYI 02960 is considered negative in this *in vivo* test system in somatic cells.

Primary Reviewer: Australia
Secondary Reviewer: Whang Phang, US EPA

B.6.4.4/02	***In Vivo* Genotoxicity (somatic cells) – Bone marrow or micronucleus**
Report:	Weiland, J. (2011). Micronucleus Assay in Bone Marrow Cells of the Mouse with BYI 02960-a.i. Harlan CCR GmbH, Rossdorf, Germany. Harlan CCR Study: 1425801. Ref: M-420536-01-2. MRID 48844135. Unpublished.
Dates of work:	12 July – 17 August 2011. Final Report 10 November 2011.
Guidelines:	OPPTS 870.5395 (1998); OECD 474 (1997); EEC 440/2008/B.12 (2008).
GLP/QA:	OECD, US EPA, Germany, EC and JMAFF GLP compliant. Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary:

BYI 02960 technical (97.2%; Lot No. PFV107N005) was evaluated in the mouse micronucleus assay for its ability to induce micronuclei in bone marrow polychromatic erythrocytes (PCEs) in female Crl:NMRI mice (7/dose level). Doses were 0, 12.5, 25 and 50 mg/kg by two intra-peritoneal injections 24 hours apart, based on the range finding results indicating 50 mg/kg bw was the maximum tolerated dose. Concurrent control groups were administered corn oil:DMSO (90:10 mixture) as the vehicle (negative) control, or 40 mg/kg of cyclophosphamide in water as the positive control. All animals were sacrificed 24 hours after the second i.p. injection. Bone marrow smears were prepared immediately after the sacrifices. In-life observations included clinical signs. Two thousand PCEs per animal were evaluated for micronuclei.

In the main study, there were no deaths, though treatment-related toxicity was observed, including ruffled fur, abdominal position, eyelid closure and reduction of spontaneous activity. No evidence of a cytotoxic effect on the bone marrow cells was observed, while the positive and vehicle controls induced the expected response. No statistically significant increases in micronucleated PCE frequency were observed in any evaluated test substance-treated group of animals.

Under the conditions of this study, BYI 02960 was neither clastogenic nor aneugenic in the mouse bone marrow micronucleus assay.

This study is classified as **reliable** (**acceptable/guideline)** and satisfies the guideline requirement (OPPTS 870.5395; OECD 474) for *in vivo* cytogenetic -erythrocyte micronucleus assay in mice mutagenicity data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   Lot/Batch #: PFV107N005
   Purity: 97.2%
   Description: Beige powder
   CAS #: 951659-40-8
   Stability of test compound: No analyses were provided investigating stability of the test material in vehicles (as indicated in the GLP declaration). The CoA indicated that the test material (as a powder) expiry date was 30 months after analysis when stored at 20±10°C.
2. Control materials
   Negative: Corn oil:DMSO mixture (90%/10%)
   Positive: Cyclophosphamide (CP) at 40 mg/kg bw in water
3. Test animals
   Species: Mouse (female only)
   Strain: Crl:NMRI
   Age at dosing: Approximately 8–9 weeks
   Weight at dosing: 28.6 ± 1.6 g
   Source: Charles River Laboratories, Sulzfeld, Germany
   Acclimation period: At least 5 days
   Diet: Pelleted standard diet (Harlan Laboratories B.V' Postbus 6174, Horst, The Netherlands)
   Water: Tap water, *ad libitum*
   Housing: Individually in Makrolon Type II cages, with granulated soft wood bedding (Rettenmaier & Söhne GmbH, Rosenberg, Germany).
4. Environmental conditions
   Temperature: 22 ± 2°C
   Humidity: 45–65%
   Air changes: Not specified
   Photoperiod: Alternating 12-hour light (6am–6pm) and dark cycles
5. Number of animals per dose
   Range-finding test: 2 females/dose
   Micronucleus assay: 7 females/dose
6 Test compound concentrations used
   Range-finding test: 10, 50, 75, 100 mg/kg bw BYI 02960 by i.p. injection
   Micronucleus assay: 0, 12.5, 25, 50 mg/kg bw BYI 02960 by i.p. injection.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

   12 July – 17 August 2011

2. Range-finding test

   Dosing concentrations used were determined by the range finding results from an experiment using groups of 2 females/dose. The highest dose used for the rangefinder for the *in vivo* micronucleus study was 100 mg/kg body weight administered by two separate i.p injections separated by 24 hours. The animals were observed for clinical signs of toxicity and mortality immediately after dosing and until at least 24 hours after the second injection.

3. Micronucleus assay

   a. Treatment and sampling times:

Test compound and vehicle control

Dosing:	☐	Once	☒	twice (24 hrs apart)	☐	Other			
Sampling (after last dose):	☐	6 hr	☐	12 hr	☒	24 hr	☐	48 hr	☐ 72 hr

Positive control

Dosing:	☒	Once	☐	twice (24 hrs apart)	☐	Other			
Sampling (after last dose):	☐	6 hr	☐	12 hr	☒	24 hr	☐	48 hr	☐ 72 hr

   b. Tissues and cells examined**:**

Bone marrow	
No. of polychromatic erythrocytes (PCE) examined per animal:	2000
No. of total erythrocytes examined per animal:	2000

4. Details of slide preparation**:** Immediately after sacrifice at 24 hours after the final injection, bone marrow was collected from at least one femur per animal, aspirated and mixed with fetal calf serum (FCS), centrifuged and placed onto slides. Bone marrow smears were stained with May-Grünwald/Giemsa stain. Prepared slides (two per sample) were coded prior to evaluation and examined for the incidence of micronucleated polychromatic erythrocytes (MPCE) and the ratio of polychromatic erythrocytes (PCE) to total normochromatic erythrocytes (NCE). 2000 PCEs were counted per animal.

5. Evaluation criteria:

The assay was considered valid and acceptable if:

- at least five animals per group could be evaluated
- the PCE to erythrocyte ration was not less than 20% of the negative control
- The positive control demonstrated a statistically significant and biologically relevant increase in mincronucleated PCEs compared to the vehicle control.

The test material was considered mutagenic if it induced either a dose-related increase, or a clear increase in the number of MPCEs in a single dose group. Biological relevance was considered as part of the evaluation. The test material was considered non-mutagenic if it did not induce a biologically relevant increase in the number of MPCEs.

6. Statistical methods:

Erythrocyte data were analysed by the non-parametric Mann-Whitney test ($p < 0.05$) as required to assist in evaluation of results.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

No analysis of test material stability was conducted in this study. The study director cited published literature data (The Extra Pharmacopoea, 29th Edition, 1967) indicating that the positive control cyclophosphamide was stable in water, with a loss of only 3.5% potency after 24 hours at 25°C.

B. RANGE-FINDING TEST

In the range-finding test, full mortality was observed in animals at 100 mg/kg bw. At 75 mg/kg bw, one animal died within the first 30 minutes of treatment, and the second animal was sacrificed due to animal welfare considerations based on the severity of clinical symptoms. No deaths were recorded at 10 or 50 mg/kg bw.

In surviving animals, clinical symptoms of toxicity included reduction of spontaneous activity, eyelid closure, ruffled fur and abdominal position *(Evaluator's note: it is unclear what this description means with respect to a clinical symptom of toxicity, though the evaluator considers that this may be a descriptor of prone posture related to the reduction of spontaneous activity*)

The study director indicated that based on the distribution of clinical symptoms and mortalities, 50 mg/kg bw was considered as the MTD under the conditions of the study. Females only were used in the micronucleus assay, noting that only male Crl:NMRI mice were used in the 2009 micronucleus study (see Herbold 2009, above).

B. MICRONUCLEUS ASSAY

1. Toxicity

No mortality occurred during the study.

No treatment-related clinical symptoms were recorded in 12.5 mg/kg bw animals.

At 25 mg/kg bw, a subset of treated female animals (1–3 out of seven) were observed with treatment-related clinical symptoms (reduction of spontaneous activity, eyelid closure, ruffled fur) at 2–4 hours after the first i.p. injection. No other clinical symptoms were observed in these animals at other time points, including after the second i.p. injection of test material.

At 50 mg/kg bw, the majority of animals (4–7 out of seven) presented with reduction of activity, abdominal position, eyelid closure and ruffled fur at 1 hour after both i.p. injections. Clinical symptoms continued through the first 2–4 hours after each treatment, but were mostly resolved apart from ruffled fur by 24 hours post-treatment.

2. PCE ratio

A very slight decrease in the PCE per 2000 erythocytes ratio was noted across treatment groups, though no dose response pattern or statistical significance was observed.

3. Micronucleated polychromatic erythrocytes (MPCEs)

No statistically significant increases in micronucleated PCE frequency were observed in any evaluated treatment group. By contrast, the positive control (CP, 40 mg/kg) induced a clear increase in MPCEs/2000 PCEs which was consistent with laboratory historical control data. Summarised results from the micronucleus assay are presented in Tables 1 and 2.

Table 1. *In vivo* micronucleus assay in female mice treated with BYI 02960: PCE frequency

Time (h)	Sex	Number of animals	Mean PCE per 2000 erythrocytes ratio				
			Vehicle control 0 mg/kg bw	Low dose 12.5 mg/kg bw	Mid dose 25 mg/kg bw	High dose 50 mg/kg bw	Positive control CP 40 mg/kg bw
24	Female	7	1229	1250	1234	1177	1125

CP = cyclophosphamide
Vehicle control = Corn oil:DMSO mixture (90:10)

Data were derived from the study report, Table 11.3 and Tables 1–5, pp. 21, 25–26

Table 2. *In vivo* micronucleus: assay in female mice treated with BYI 02960: MPCE frequency

Time (h)	Sex	Number of animals	Mean % micronucleated PCE/2000 PCE (range)				
			Vehicle control 0 mg/kg bw	Low dose 12.5 mg/kg bw	Mid dose 25 mg/kg bw	High dose 50 mg/kg bw	Positive control CP 40 mg/kg bw
24	Female	7	0.121 (0–5)	0.114 (1–5)	0.143 (1–5)	0.093 (0–3)	2.264 (32–63)

CP = cyclophosphamide
Vehicle control = corn oil:DMSO mixture (90:10)
() = Range

Data were derived from the study report, Table 11.3 and Tables 1–5, pp. 21, 25–26

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 474 (1997) and other equivalent guidelines.

C. CONCLUSIONS:

BYI 02960 did not induce an increase in micronuclei in bone marrow cells of mice at doses up to the maximum tolerated dose of 50 mg/kg bw (as defined by the study director from a range-finding study). The sensitivity of the test system to detect a biologically relevant increase in MPCEs was adequately demonstrated by the results obtained with the positive control, CP and comparison with historical control data.

Accordingly, BYI 02960 is considered negative in this *in vivo* test system in somatic cells.

IIA 6.4.5 Summary of Genotoxicity of BYI 02960

BYI 02960 has been examined in a range of *in vitro* and *in vivo* genotoxicity assays, including endpoints of gene mutation and chromosomal damage. *In vitro*, BYI 02960 was negative for gene mutation in bacteria (Ames test) and mammalian cells (HPRT locus, Chinese hamster V79 cells) with and without metabolic activation. In the *in vitro* cytogenetic assay using Chinese hamster V79 cells, BYI 02960 did not induce chromosomal aberrations with and without metabolic activation.

In vivo, BYI 02960 was found to be non-clastogenic in the rat bone marrow micronucleus assay in male and female rats, with both studies being conducted up to and including the maximum tolerated dose.

Table B.6.4.5.1: Summary of Genotoxicity Studies

Study	**Dose Levels**	**Result**
***In vitro* studies**		
Bacterial reverse mutation Herbold, B (2009)	16-5000 µg/plate	Negative ± S9
Bacterial reverse mutation Sokolowski, A (2011)	3 -5000 µg/plate	Negative ± S9
Mammalian cell gene mutation Entian, G (2009)	46-2944 µg/mL	Negative ± S9
In vitro cytogenetics Thum, M (2009)	200 – 3000 µg/mL	Negative ± S9
***In vivo* studies**		
Mouse bone marrow micronucleus (Male mice, i.p.) Herbold, B (2009b)	10 - 40 mg/kg bw	Negative
Mouse bone marrow micronucleus (Female mice, i.p.) Weiland, J (2011)	12.5 - 50 mg/kg bw	Negative

Primary review: Australia
Secondary review: US EPA &PMRA

B.6.8.3/16 ***In Vivo* Genotoxicity (somatic cells) – Bone marrow or micronucleus**

Report:	Hall, C. (2011b) Micronucleus Assay in Bone Marrow Cells of the Mouse with BYI 02960-difluoroethyl-aminofuranone (metabolite of BYI 02960). Harlan CCR Study: 1421401. Ref: M-420540-01-2. MRID 48844136. Unpublished.
Dates of work:	5 July – 9 August 2011. Final Report 28 November 2011.
Guidelines:	OPPTS 870.5395 (1998); OECD 474 (1997); EEC 440/2008/B.12 (2008).
GLP/QA:	OECD, US EPA, Germany, EC and JMAFF GLP compliant. Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary:

BYI 02960-difluoroethyl-aminofuranone (98.5%; Lot No. NLL 8671-6-1) was evaluated in the mouse micronucleus assay for its ability to induce micronuclei in bone marrow polychromatic erythrocytes (PCEs) in male Crl:NMRI mice (7/dose level). Doses were 0, 125, 250 and 500 mg/kg by two intra-peritoneal injections 24 hours apart, based on the range finding results indicating 500 mg/kg bw was the maximum tolerated dose. Concurrent control groups were administered water as the vehicle (negative) control, or 40 mg/kg of cyclophosphamide in water as the positive control. All animals were sacrificed 24 hours after the second i.p. injection. Bone marrow smears were prepared immediately after the sacrifices. In-life observations included clinical signs. Two thousand PCEs per animal were evaluated for micronuclei.

In the main study, there were no deaths, though treatment-related toxicity was observed, including ruffled fur, abdominal position, eyelid closure and reduction of spontaneous activity. No evidence of a cytotoxic effect on the bone marrow cells was observed, while the positive and vehicle controls induced the expected response. No statistically significant increases in micronucleated PCE frequency were observed in any evaluated test substance-treated group of animals.

Under the conditions of this study, BYI 02960-difluoroethyl-aminofuranone was neither clastogenic nor aneugenic in the mouse bone marrow micronucleus assay.

This study is classified as **reliable** (**acceptable/guideline)** and satisfies the guideline requirement (OPPTS 870.5395; OECD 474) for *in vivo* cytogenetic -erythrocyte micronucleus assay in mice mutagenicity data.

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	BYI 02960-difluoroethyl-aminofuranone
	Lot/Batch #:	NLL 8671-6-1
	Purity:	98.5%
	Description:	White powder
	CAS #:	Not specified
	Stability of test compound:	No analyses were provided investigating stability of the test material in vehicle (as indicated in the GLP declaration). The CoA indicated that the test material (as a powder) expiry date was six months after analysis when stored at 20±10°C.
2.	Control materials	
	Negative:	Water (sterilised)
	Positive:	Cyclophosphamide (CP) at 40 mg/kg bw in water
3.	Test animals	
	Species:	Mouse (male only)
	Strain:	Crl:NMRI
	Age at dosing:	Approximately 9–10 weeks
	Weight at dosing:	28.6 ± 1.6 g
	Source:	Charles River Laboratories, Sulzfeld, Germany
	Acclimation period:	At least 5 days
	Diet:	Pelleted standard diet (Harlan Laboratories B.V' Postbus 6174, Horst, The Netherlands)
	Water:	Tap water, *ad libitum*
	Housing:	Individually in Makrolon Type II cages, with granulated soft wood bedding (Rettenmaier & Söhne GmbH, Rosenberg, Germany).
4.	Environmental conditions	
	Temperature:	22 ± 2°C
	Humidity:	45–65%
	Air changes:	Not specified
	Photoperiod:	Alternating 12-hour light (6am–6pm) and dark cycles
5.	Number of animals per dose	
	Range-finding test:	2/sex/dose
	Micronucleus assay:	7 males/dose
6	Test compound concentrations used	
	Range-finding test:	500, 750, 1000 mg/kg bw (+ additional 500 mg/kg bw test group for males only) by i.p. injection
	Micronucleus assay:	0, 125, 250, 500 mg/kg bw by i.p. injection.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

5 July – 9 August 2011

2. Range-finding test

Dosing concentrations used were determined by the range finding results from an experiment using groups of 2 animals/sex/dose. The highest dose used for the rangefinder for the *in vivo* micronucleus study was 1000 mg/kg body weight administered by two separate i.p injections separated by 24 hours. The animals were observed for clinical signs of toxicity and mortality immediately after dosing and until at least 24 hours after the second injection.

3. Micronucleus assay

a. Treatment and sampling times:

Test compound and vehicle control

Dosing:	☐	Once	☒	twice (24 hrs apart)	☐	Other			
Sampling (after last dose):	☐	6 hr	☐	12 hr	☒	24 hr	☐	48 hr	☐ 72 hr

Positive control

Dosing:	☒	Once	☐	twice (24 hrs apart)	☐	Other			
Sampling (after last dose):	☐	6 hr	☐	12 hr	☒	24 hr	☐	48 hr	☐ 72 hr

b. Tissues and cells examined**:**

Bone marrow	
No. of polychromatic erythrocytes (PCE) examined per animal:	2000
No. of total erythrocytes examined per animal:	2000

4. Details of slide preparation**:** Immediately after sacrifice at 24 hours after the final injection, bone marrow was collected from at least one femur per animal, aspirated and mixed with fetal calf serum (FCS), centrifuged and placed onto slides. Bone marrow smears were stained with May-Grünwald/Giemsa stain. Prepared slides (two per sample) were coded prior to evaluation and examined for the incidence of micronucleated polychromatic erythrocytes (MPCE) and the ratio of polychromatic erythrocytes (PCE) to total normochromatic erythrocytes (NCE). 2000 PCEs were counted per animal.

5. Evaluation criteria:

The assay was considered valid and acceptable if:

- at least five animals per group could be evaluated
- the PCE to erythrocyte ration was not less than 20% of the negative control
- The positive control demonstrated a statistically significant and biologically relevant increase in mincronucleated PCEs compared to the vehicle control.

The test material was considered mutagenic if it induced either a dose-related increase, or a clear increase in the number of MPCEs in a single dose group. Biological relevance was considered as part of the evaluation. The test material was considered non-mutagenic if it did not induce a biologically relevant increase in the number of MPCEs.

6. Statistical methods:

Erythrocyte data were analysed by the non-parametric Mann-Whitney test ($p < 0.05$) as required to assist in evaluation of results.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

No analysis of test material stability was conducted in this study. The study director cited published literature data (The Extra Pharmacopoea, 29th Edition, 1967) indicating that the positive control cyclophosphamide was stable in water, with a loss of only 3.5% potency after 24 hours at 25°C.

B. RANGE-FINDING TEST

In the range-finding test, full mortality was observed in animals at 750 and 1000 mg/kg bw within 2–4 hours after the first i.p. injection of test material. No deaths were recorded at 500 mg/kg bw. Treatment-related clinical symptoms included reduction of spontaneous activity, eyelid closure, ruffled fur and abdominal position in both sexes. *(Evaluator's note: it is unclear what this description means with respect to a clinical symptom of toxicity, though the evaluator considers that this may be a descriptor of prone posture related to the reduction of spontaneous activity*)

The study director indicated that based on the distribution of clinical symptoms and mortalities, 500 mg/kg bw was considered as the MTD under the conditions of the study. Additionally, the study director noted that there were no substantial sex-specific differences between male and female animals at 500 mg/kg bw. On this basis, it was decided that the main study would use only male animals.

B. MICRONUCLEUS ASSAY

1. Toxicity

No mortality occurred during the study.

No treatment-related clinical symptoms were recorded in 125 mg/kg bw animals.

At 250 mg/kg bw, a subset of treated male animals (3–7 out of seven) were observed with treatment-related clinical symptoms (reduction of spontaneous activity, eyelid closure, abdominal position, ruffled fur) at 1 hour after the first i.p. injection. Clinical symptoms resolved by 24 hours after the first i.p. injection, with only a single incidence of abdominal position observed in one animal after the second i.p. injection of test material. No other clinical symptoms were observed in these animals at other time points.

At 500 mg/kg bw, the majority of animals (5–7 out of seven) presented with reduction of activity, abdominal position, eyelid closure and ruffled fur for up to six hours after the first i.p. injection, with resolution of symptoms at 24 hours (i.e. prior to the second i.p. injection of test material).

After the second administration of test material at 500 mg/kg bw, a similar pattern of clinical symptoms was observed during the first four hours after treatment, though no treatment-related incidence of ruffled fur was noted at any time point after the second i.p. injection. Clinical symptoms continued through the first 2–4 hours after each treatment, but were mostly resolved by 24 hours post-treatment.

2. PCE ratio

A very slight decrease in the PCE per 2000 erythocytes ratio was noted across treatment groups, though no dose response pattern or statistical significance was observed, suggesting a lack of cytotoxicity.

3. Micronucleated polychromatic erythrocytes (MPCEs)

A dose-related increase in the frequency of MPCEs per 2000 PCEs was observed when compared with concurrent negative controls (Table 1). However, all reported values were within the mean (0.04 – 0.25) and minimum/maximum range values (0 – 12) of historical control data for negative (vehicle) controls. By contrast, the positive control (CP, 40 mg/kg) induced a clear increase in MPCEs/2000 PCEs which was consistent with laboratory historical control data. Summarised results from the micronucleus assay are presented in Tables 1 and 2.

Table 1. *In vivo* micronucleus assay in male mice treated with BYI 02960-difluoroethyl-aminofuranone: PCE frequency

Time (h)	Sex	Number of animals	Mean PCE per 2000 erythrocytes ratio				
			Vehicle control 0 mg/kg bw	Low dose 125 mg/kg bw	Mid dose 250 mg/kg bw	High dose 500 mg/kg bw	Positive control CP 40 mg/kg bw
24	Male	7	1158	1120	1118	1046	1039

CP = cyclophosphamide
Vehicle control = water

Data were derived from the study report, Table 11.3 and Tables 1–5, pp. 21, 25–26

Table 2. *In vivo* micronucleus: assay in male mice treated with BYI 02960-difluoroethyl-aminofuranone: MPCE frequency

Time (h)	Sex	Number of animals	Mean % micronucleated PCE/2000 PCE (range)				
			Vehicle control 0 mg/kg bw	Low dose 125 mg/kg bw	Mid dose 250 mg/kg bw	High dose 500 mg/kg bw	Positive control CP 40 mg/kg bw
24	Male	7	0.100 (0–4)	0.086 (0–4)	0.114 (1–3)	0.164 (2–7)	1.964 (28–55)

CP = cyclophosphamide
Vehicle control = water
() = Range

Data were derived from the study report, Table 11.3 and Tables 1–5, pp. 21, 25–26

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 474 (1997) and other equivalent guidelines.

C. CONCLUSIONS:

BYI 02960-difluoroethyl-aminofuranone did not induce an increase in micronuclei in bone marrow cells of mice at doses up to the maximum tolerated dose of 500 mg/kg bw (as defined by the study director from a range-finding study). The sensitivity of the test system to detect a biologically relevant increase in MPCEs was adequately demonstrated by the results obtained with the positive control, CP and comparison with historical control data.

Accordingly, BYI 02960-difluoroethyl-aminofuranone is considered negative in this *in vivo* test system in somatic cells.

Primary review: Australia
Secondary review: US EPA & PMRA

B.6.8.3/17 *In Vivo* Unscheduled DNA Synthesis

Report:	Hall, C. (2011c) *In vivo* Unscheduled DNA Synthesis in Rat Hepatocytes with BYI 02960-difluoroethyl-aminofuranone (metabolite of BYI 02960). Harlan CCR Study: 1421402. Ref: M-420111-01-2. MRID 48844137. Unpublished.
Dates of work:	5 July – 31 August 2011. Final Report 26 October 2011.
Guidelines:	OPPTS 870.5550 (1998); OECD 486 (1997); EEC 2000/32, B.39 (2000).
GLP/QA:	OECD, US EPA, Germany, EC and JMAFF GLP compliant. Quality Assurance and Data Confidentiality statements were provided.

Executive Summary:

BYI 02960-difluoroethyl-aminofuranone (98.5%; Lot No. NLL 8671-6-1) was evaluated in the *in vivo* unscheduled DNA synthesis assay in male rat heptaocytes for its ability to induce DNA repair. Doses administered to male Wistar rats (4/dose/sacrifice time) were 0, 1000 and 2000 mg/kg by a single oral gavage dose, based on the range finding results indicating 2000 mg/kg bw was the maximum tolerated dose. Concurrent control groups were administered water as the vehicle (negative) control, 80 mg/kg of N,N'-dimethylhydrazinedihydrochloride (DMH; 4-hour preparations) or 2-acetylaminofluorene (2-AAF; 16-hour preparations) in water as the positive control. Hepatocyte cells were extracted by perfusion from animals at 4 hours or 16 hours after treatment and exposed to methyl-^{3}H-thymidine for 4 hours, then processed to determine the extent of unscheduled DNA synthesis.

BYI 02960-difluoroethyl-aminofuranone did not induce an increase in *in vivo* unscheduled DNA synthesis in male rat hepatocytes at doses up to and including the limit dose (also the maximum tolerated dose) of 2000 mg/kg bw. As no sex-specific differences in the response were noted in the range-finding study, a similar lack of response is considered likely in females. The sensitivity of the test system to detect a biologically relevant increase in unscheduled DNA synthesis was adequately demonstrated by the results obtained with the positive controls in both 4-hour (DMH) and 16-hour (2-AAF) preparations of rat hepatocytes.

Under the conditions of this study, BYI 02960-difluoroethyl-aminofuranone did not induce an increase in unscheduled DNA synthesis at up to the limit dose of 2000 mg/kg bw.

This study is classified as **reliable** (**acceptable/guideline)** and satisfies the guideline requirement (OPPTS 870.5550; OECD 486) for an *in vivo* unscheduled DNA synthesis assay in rat hepatocytes.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BYI 02960-difluoroethyl-aminofuranone
Lot/Batch #:	NLL 8671-6-1
Purity:	98.5%
Description:	White powder
CAS #:	Not specified
Stability of test compound:	No analyses were provided investigating stability of the test material in vehicle (as indicated in the GLP declaration). The CoA indicated that the test material (as a powder) expiry date was six months after analysis when stored at 20±10°C.
2. Control materials	
Negative:	Water (sterilised)
Positive:	N, N'-dimethylhydrazinedihydrochloride (DMH) at 80 mg/kg bw in 0.9% NaCl solution (4-hour preparation) 2-acetylaminofluorene (2-AAF) at 100 mg/kg bw in DMSO/PEG 400 (10:90) mixture.
3. Test animals	
Species:	Rat (male only)
Strain:	Wistar
Age at dosing:	Approximately 6 weeks
Weight at dosing:	181.7 ± 13.2 g
Source:	Harlan Laboratories B.V., Horst, The Netherlands
Acclimation period:	5 days
Diet:	Pelleted standard diet (Harlan Laboratories B.V. Postbus 6174, Horst, The Netherlands)
Water:	Tap water, *ad libitum*
Housing:	Group-housed (size not specified) in Makrolon Type III/IV cages, with granulated soft wood bedding (Rettenmaier & Söhne GmbH, Rosenberg, Germany).
4. Environmental conditions	
Temperature:	22 ± 2°C
Humidity:	45–65%
Air changes:	Not specified
Photoperiod:	Alternating 12-hour light (6am–6pm) and dark cycles
5. Number of animals per dose	
Range-finding test:	2/sex/dose
Micronucleus assay:	4 males/dose/sacrifice time
6 Test compound concentrations used	
Range-finding test:	2000 mg/kg bw by oral gavage (limit dose)

Unscheduled DNA Synthesis assay:	1000 and 2000 mg/kg bw by oral gavage.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

5 July – 31 August 2011

2. Range-finding test (*in vivo* phase)

The highest dose used for the rangefinder test for the unscheduled DNA synthesis assay was the limit dose of 2000 mg/kg body weight administered once by oral gavage. The animals (2/sex) were observed for clinical signs of toxicity and mortality immediately after dosing and at 1, 2–4, 6 and 24 hours after administration of the test dose. Based on the results of this preliminary study, only male rats were used in the main UDS test.

3. Main unscheduled DNA synthesis assay

a. Treatment and sampling times (*in vivo* phase):

Test compound and vehicle control

Dosing (by oral gavage):	[×]	Once	[ ]	twice (24 hrs apart)	[ ]	Other			
Sampling (after last dose):	[×]	4 hr	[×]	16 hr	[ ]	24 hr	[ ]	48 hr	[ ] 72 hr

Positive controls

Dosing (by oral gavage):	[×]	Once	[ ]	twice (24 hrs apart)	[ ]	Other			
Sampling (after last dose):	[×]	4 hr	[×]	16 hr	[ ]	24 hr	[ ]	48 hr	[ ] 72 hr

b. Treatment and sampling times (*in vitro* phase)

At *in vivo* phase termination, animals were anaesthetised with a ketamine/xylazin/midazolam mixture (46:23:31 ratio). Livers were perfused through the *vena portae* with HBSS (pH 7.4) supplemented with 0.05 % (w/v) collagenase, with solutions maintained at 37°C. Isolated hepatocytes were washed and processed to yield a single cell suspension, then transferred into Williams Medium E (WME), pH 7.6, supplemented with HEPES (2.38 mg/mL), penicillin (100 U/mL), streptomycin (0.1 mg/mL), L-glutamine (0.29 mg/mL), insulin (0.5 µg/mL) and FCS (100 µL/mL) [hereafter referred to as complete medium]. Cell suspensions were plated at least in triplicate into dishes and incubated for 1.5 hours at 37°C to allow for cell attachment. After this attachment period, cells were washed and the incubated with ^{3}HTdR (5 µCi/mL) for 4 hours, then washed and incubated with unlabelled thymidine overnight.

4. Details of slide preparation**:**

After incubation with unlabelled thymidine, cells were then washed, harvested and mounted onto slides for autoradiographic processing, with slides coated with

photographic emulsion (Kodak NTB) in the dark for 14 days at 4°C. After incubation, the photographic emulsion was developed, then fixed and stained with hematoxylin/eosin.

Evaluation of cells was performed microscopically. The number of silver grains in the nuclear area of cells was counted using automated equipment. Additionally, the number of silver grains of the most heavily labelled nuclear-sized cytoplasm area adjacent to the nucleus was counted. At least two slides per animal and 50 cells per slide were evaluated. Heavily radiolabelled cells undergoing replicative DNA synthesis were excluded from counting.

5. Evaluation criteria:

Nuclear and net grain counts were estimated together. As defined by the study director, increased net grain counts should be based on enhanced nuclear grain counts rather than on decreased cytoplasmic grain counts.

The test material was classified as inducing a positive response where the mean number of net grains was >5 per nucleus at one of the test points.

A group average between 0–5 net grains per nucleus was classified as a marginal response. Where a marginal response was noted, examination of whether a dose-related increase in nuclear and net grains and/or a substantial shift of the percentage distribution of the nuclear grain counts to higher values was relied on to support classification of the test material as a positive inducer of unscheduled DNA synthesis. Supportive evidence was also drawn from statistical analyses.

A result of < 0 net grains at any of the test points was considered negative (i.e. the test material was non-effective in the test system).

6. Statistical methods:

Quantitative data were evaluated using the non-parametric Mann-Whitney test where appropriate.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

No analysis of test material stability was conducted in this study. Two separate analyses of the same batch of test material six months apart indicated that no degradation of the test material had occurred.

B. RANGE-FINDING TEST

In the range-finding test, no mortalities were observed at the limit dose of 2000 mg/kg bw. Clincial signs of toxicity included reductions in spontaneous activity and ruffled fur

throughout the 24-hour observation period, and eyelid closure between 2–6 hours after test material administration.

The study director indicated that based on the distribution of clinical symptoms and mortalities, the limit dose of 2000 mg/kg bw was considered as the MTD under the conditions of the study. Additionally, the study director noted that there were no substantial sex-specific differences between male and female animals. On this basis, it was decided that the main study would use only male animals.

B. MAIN ASSAY

1. Toxicity (*in vivo* phase)

No mortality occurred during the study.

At both 1000 and 2000 mg/kg bw, clear signs of treatment-related toxicity were noted, consisting of ruffled fur and reduction in spontaneous activity. Overall, symptoms were observed at both doses up to the point of animal sacrifice.

2. Grain counts (nucleus, cytoplasmic area, net)

As shown in Tables 1 and 2, no clear evidence of a dose-related change in the mean net grain counts was observed at both cell preparation (i.e. sacrifice) time points (4 hours and 16 hours). The mean number of nuclear grains of cells in repair was also similar between negative controls and treatment groups. The positive controls at the two time points demonstrated a clear increase in the mean net grain counts (a positive value, compared with the negative values observed in negative vehicle controls and treatment groups), and reported positive control results were similar to values reported in historical control data provided by the study director.

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 486 (1997) and other equivalent guidelines.

C. CONCLUSIONS:

BYI 02960-difluoroethyl-aminofuranone did not induce an increase in *in vivo* unscheduled DNA synthesis in male rat hepatocytes at doses up to the limit dose (also the maximum tolerated dose) of 2000 mg/kg bw. As no sex-specific differences in the response were noted in range-finding studies, a similar lack of response is likely in females. The sensitivity of the test system to detect a biologically relevant increase in unscheduled DNA synthesis was adequately demonstrated by the results obtained with the positive controls in both 4-hour (DMH) and 16-hour (2-AAF) preparations of rat hepatocytes.

Accordingly, BYI 02960-difluoroethyl-aminofuranone is considered negative in this *in vivo* test system in somatic cells.

Table 1. *In vivo* UDS assay in male mice treated with BYI 02960-difluoroethyl-aminofuranone: 4-hour preparation samples

Dose group (mg/kg bw)	Animal ID	Mean nuclear grain count	Mean cytoplasmic grain count	Mean net grain count	Mean nuclear grains of cells in repair	% cells in repair
0 (vehicle control)	1	12.37 ± 6.56	18.92 ± 7.92	-6.55 ± 6.12	7.33 ± 1.53	3
	2	11.56 ± 5.87	19.46 ± 6.62	-7.90 ± 5.69	9.00 ± 0.00	2
	3	14.88 ± 6.06	27.96 ± 10.16	-13.08 ± 8.54	0 ± 0.00	0
	4	11.30 ± 5.98	20.09 ± 10.45	-8.79 ± 9.86	10.00 ± 7.07	2
	Average	**12.53 ± 6.12**	**21.61 ± 8.79**	**-9.08 ± 7.07**	**6.58 ± 2.15**	**1.75**
1000	5	13.74 ± 15.58	22.70 ± 20.09	-8.96 ± 9.86	6.80 ± 2.49	5
	6	10.05 ±3.98	17.66 ± 5.20	-7.61 ± 5.39	0 ± 0.00	0
	7	15.92 ± 6.35	23.39 ± 8.22	-7.47 ± 6.65	6.50 ± 0.71	2
	8	13.01 ± 5.44	24.07 ± 8.08	-11.06 ± 7.72	11.00 ± 0.00	1
	Average	**13.18 ± 7.84**	**21.96 ± 10.40**	**-8.78 ± 7.40**	**6.08 ± 0.80**	**2.00**
2000	9	12.15 ± 4.67	21.57 ± 5.88	-9.42 ± 6.81	0 ± 0.00	0
	10	17.15 ± 7.86	27.36 ± 9.81	-10.21 ± 8.07	8.33 ± 3.06	3
	11	43.06 ± 17.55	59.53 ± 22.10	-16.47 ± 13.71	9.25 ± 2.50	4
	12	19.23 ± 11.67	29.28 ± 16.93	-10.05 ± 8.08	6.33 ± 0.58	3
	Average	**22.90 ± 10.44**	**34.44 ± 13.68**	**-11.54 ± 9.17**	**5.98 ± 1.53**	**2.50**
80 (DMH) Positive control	13	46.91 ± 18.57	14.17 ± 5.88	32.74 ± 15.84	33.05 ± 15.61	99
	14	67.28 ± 19.63	17.87 ± 6.71	49.41 ± 18.41	49.41 ± 18.41	100
	15	55.58 ± 13.17	27.91 ± 9.20	27.67 ± 12.02	27.67 ± 12.02	100
	16	57.73 ± 23.86	20.57 ± 7.22	37.16 ± 23.21	40.45 ± 21.15	92
	Average	**56.88 ± 18.81**	**20.13 ± 7.25**	**36.74 ± 17.37**	**37.64 ± 16.80**	**97.75**

Table 2. *In vivo* UDS assay in male mice treated with BYI 02960-difluoroethyl-aminofuranone: 16-hour preparation samples

Dose group (mg/kg bw)	Animal ID	Mean nuclear grain count	Mean cytoplasmic grain count	Mean net grain count	Mean nuclear grains of cells in repair	% cells in repair
0 (vehicle control)	17	15.17 ± 6.05	30.39 ± 9.21	-15.22 ± 7.62	7.00 ± 0.00	1
	18	12.98 ± 5.57	24.07 ± 8.81	-11.09 ± 7.50	0 ± 0	0
	19	19.81 ± 8.98	32.80 ± 13.40	-12.99 ± 9.40	5.00 ± 0.00	2
	20	11.87 ± 5.77	20.74 ± 7.15	-8.87 ± 6.30	13.00 ± 0.00	1
	Average	**14.96 ± 6.59**	**27.00 9.64**	**-12.04 ± 7.70**	**6.25 ± 0.00**	**1.00**
1000	21	35.74 ± 13.50	45.41 ± 17.04	-9.67 ± 10.76	8.86 ± 2.34	7
	22	25.89 ± 14.32	35.42 ± 18.12	-9.53 ± 8.27	9.00 ± 0.00	1
	23	20.15 ± 7.39	30.38 ± 11.26	-10.23 ± 8.68	11.00 ± 0.00	1
	24	20.78 ± 8.01	30.73 ± 10.14	-9.95 ± 8.97	6.50 ± 1.00	4
	Average	**25.64 ± 10.81**	**35.48 ± 14.14**	**-9.84 ± 9.17**	**8.84 ± 0.84**	**3.25**
2000	25	14.36 ± 6.35	29.01 ± 9.72	-14.65 ± 9.28	0 ± 0	0
	26	19.37 ± 8.90	36.37 ± 13.08	-17.00 ± 10.20	0 ± 0	0
	27	17.15 ± 6.48	31.43 ± 6.67	-14.28 ± 8.39	8.00 ± 0.00	1
	28	11.58 ± 5.19	21.85 ± 6.75	-10.27 ± 6.52	5.00 ± 0.00	1
	Average	**15.61 ± 6.73**	**29.67 ± 9.06**	**-14.05 ± 8.60**	**3.25 ± 0.00**	**0.50**
100 (2-AAF) Positive control	29	30.63 ± 12.46	13.80 ± 8.39	16.83 ± 10.08	18.19 ± 8.92	93
	30	34.38 ± 10.82	21.41 ± 8.97	12.97 ± 8.16	14.85 ± 7.11	86
	31	43.39 ± 10.82	31.23 ± 9.59	12.16 ± 7.88	14.21 ± 6.26	86
	32	64.54 ± 11.94	50.49 ± 11.14	14.05 ± 8.83	16.76 ± 6.76	83
	Average	**43.24 ± 11.51**	**29.23 ± 9.52**	**14.00 ± 8.74**	**16.00 ± 7.26**	**87.00**

Primary review: Australia
Secondary review: US EPA & PMRA

Report: Garcin JC (2011); BYI 02960 - An acute neurotoxicity study in the rat by oral administration. Bayer CropScience, 355, rue Dostoi'evski, BP153, 06903 Sophia Antipolis Cedex, France. Study No. SA 10096. Sponsor identification number: Lynx-PSI N°TXRVP026. Bayer CropScience File No. M-415408-01-2. 30 September. Unpublished. PMRA 2239240. MRID 48844138.

Guidelines: OECD 424 (1997); OPPTS 870.6200 (1998); MAFF No. 8147 (2000)
Deviations: None

GLP: Yes Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In an acute neurotoxicity study (MRID 48844138), Wistar (Rj:WI (IOPS HAN)) rats in groups of 12/sex/dose received BYI 02960 (96.2%; 2009-000239) at 0, 50, 200, or 800 mg/kg bw/day as single oral gavage dose in methylcellulose 400 (0.5% v/v) at 10 mL/kg bw final dosing volumes. A follow up cohort of 12 females/dose received 0, 20, or 35 mg/kg bw/day oral gavage doses.

Neurobehavioural parameters examined in the first cohort consisted of motor activity and functional observational battery (FOB) assessments at 2 hours post dosing and on Days 7 and 14 after treatment, along with neuropathological examinations of the peripheral and central nervous systems at study termination.

There were two instances of unscheduled mortality in females at 800 mg/kg bw/day, with animals found dead on study Days 1 (day of dosing) and Day 5, respectively. Both mortalities were test substance related. Clinical signs prior to death in the animal found dead on study day 1 consisted of piloerection, low muscle tone and arousal, rapid respiration, tremors, myoclonic jerks, convulsions, dilated pupils, absence of pupil and flexor reflexes and uncoordinated or slow surface righting reflex.

Effects on bodyweight gain were confined to 40/46% (M/F) lower mean bodyweight gains at 800 mg/kg bw/day in study week 1 relative to controls that subsequently resolved such that by study day 14 there were no differences in group mean bodyweight.

Changes in FOB and activity parameters were confined to study day 1 only, with no treatment-related findings being present on study days 7 or 14.

At 50 mg/kg bw/day, increased incidences of piloerection in males and females (8/12 for M and 7/12 for F, vs 4/12 for both M and F controls) and of dilated pupils in females (6/12, vs 1/12 for controls) were observed on study day 1.

At 200 mg/kg bw/day/day, piloerection, dilated pupils, rapid respiration, lower muscle tone, low arousal, repetitive licking of lips, decreased rearing, exaggerated flexor reflexes, gait incoordination and flattened body posture, and higher incidence of tremors were seen in both sexes. In addition, males from the 200 mg/kg bw/day/day dose group exhibited myoclonic jerks and absence of movement, and were also cold to touch, while a higher incidence of walking on tiptoes was observed in females at this dose. Automated measures of motor activity were reduced during the first 10-min interval of the session in both sexes.

At 800 mg/kg bw/day/day, piloerection, lower muscle tone, rapid respiration, low arousal, tremors, myoclonic jerks, chewing, repetitive licking of lips, gait incoordination, flattened or hunched posture, dilated pupils, impaired (uncoordinated or slow) righting reflex, impaired flexor and tail pinch responses and reduced rectal temperature were observed. Automated measures of motor activity were also reduced in both sexes.

In the follow-up study, the animals at 20 and 35 mg/kg showed no treatment-related effects in all the parameters examined.

No gross or microscopic test substance-related morphological changes were seen in the nervous system tissues.

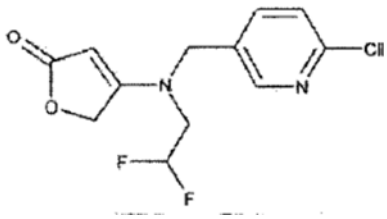
The NOAEL in this acute neurotoxicity study is 35 mg/kg bw/day/day; LOAEL is 50 mg/kg bw/day/day based on increased incidences of piloerection in both sexes and increased incidence of pupil dilation in females on study day 1.

This study is fully reliable (acceptable/guideline) and satisfies the data requirements for an acute neurotoxicity study (OPPTS 870.6200; OECD 424).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flupyradifurone (BYI 02960)
   4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one



Batch #:	2009-000239
Purity:	96.2% (1H-NMR)
Description:	beige powder
CAS #:	951659-40-8
Stability of test compound:	Stated to be stable for two years if stored at 25±3°C (date of analysis: 14 Jan 2011). Once diluted in methylcellulose 400 (0.5%) at 1 or 250 g/L, the test substance was found to be stable at up to 28 days under similar storage conditions. The achieved concentrations of the dilutions were found to be within 95-101% of the nominal concentrations. The homogeneity of the test substance was confirmed at the highest and lowest dosing concentrations in the initial study, and at both the concentrations tested in the follow-up study (95-103% nominal concentrations).

2. Vehicle: methylcellulose 400 (0.5% v/v)
3. Test animals

Species:	Rat
Strain:	Wistar (Rj:WI (IOPS HAN))
Age at dosing:	7 to 8 weeks
Weight at dosing (mean):	261 to 356g for males; 183 to 257 g for females; or (study 2) 173 to 214 g (females)
Source:	R. Janvier, Le Genest St Isle, France
Acclimation period:	At least 14 days
Diet:	A04C-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum* except during neurobehavioural testing.
Water:	Filtered and softened tap water, *ad libitum* except during neurobehavioural testing.
Housing:	Individually in suspended, stainless steel, wire mesh cages.

4. Environmental conditions

Temperature:	20 to 24°C

Humidity:	40 to 70%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

   04 May 2010 to 03 March 2011

2. Animal assignment and treatment

   Animals were assigned to the test groups noted in Table KIIA 5.7.1/01. Non-fasted rats in groups of 12/sex/dose received a single oral gavage dose of flupyradifurone (BYI 02960) at 0, 50, 200, or 800 mg/kg bw/day in methylcellulose 400 (0.5%) at a final dosing volume of 10 mL/kg bw and were observed for 14 days post-dosing. A follow-up study was conducted with 12 females/dose receiving single oral doses of 0, 20 or 35 mg/kg bw/day in the same vehicle; these animals were sacrificed after neurobehavioural examinations on the day of dosing.

   Animals were assigned to dose groups by computerised, stratified randomisation so that all individuals had a bodyweight within 20% of the mean for that group on the day of randomisation. The negative control groups received the vehicle only.

   The initial dosing regimen was chosen based on the results of acute oral toxicity study in rats (Gilissen, 2009; Study No. AT05287) and preliminary study on time of peak effect in rats (Garcin, 2011; Study No. SA10178). The follow-up study dosing regimen was selected on the basis of piloerection and pupil dilatation being present at the lowest dose tested of 50 mg/kg bw/day in the initial study. Only females were tested in the follow-up study since they were equal or more sensitive than males at higher dose levels.

Table KIIA 5.7.1/01: Study Design

No./ Sex/Dose	Dose (mg/kg bw/day)	Animal No's	
		M	F
12	0	UT1M2393 to 2404	UT1F2405 to 2416
12 F only	0 (b)	-	VT5F0827 to 0838
12 F only	20 (b)	-	VT6F0839 to 0850
12 F only	35 (b)	-	VT7F0851 to 0862
12	50	UT2M2417 to 2428	UT2F2429 to 2440
12	200	UT3M2441 to 2452	UT3F2453 to 2464
12	800	UT4M2465 to 2476	UT4F2477 to 2488

Dosing vehicle was 0.5% (v/v) methylcellulose 400, and final dosing volume was 10 mL/kg bw. '(b)' – denotes follow-up study groups. Data excerpted from page 18 of the study report.

3. Dosing formulation and analysis

   One batch of test substance in vehicle was prepared at each concentration of 2, 3.5, 5, 20, and 80 g/L during the study, corresponding to the nominal dosing levels of 20, 35, 50, 200, and 800 mg/kg bw/day. The 5 to 80 g/L solutions were formulated on 21 May 2010 and used over 25 to 28 May, and the 2 and 3.5 g/L solutions were formulated on 25 or 28 February 2011 and used on 2 March 2011. When not in use, the formulations were stored at approximately 5±3°C. Homogeneity of test substance concentrations were analysed by

HPLC-UV for all batches and reported to be 95 to 103% of nominal concentrations. Test substance purity was not accounted for when calculated achieved test substance concentrations or achieved doses.

The stability of the test substance in 0.5% methylcellulose 400 suspension at concentrations of 1 and 250 g/L was stated to have been demonstrated for up to 28 days under similar conditions in another study by the laboratory (Amir Tahmasseb, 2007; Study No. SA07025).

Based on this information, it can be concluded that the animals received doses sufficiently comparable to the targeted dose levels during the study.

4. Statistics

Statistical analyses were undertaken on bodyweights, organ weights, and FOB parameters using the Path/Tox System V4.2.2 (Table KIIA 5.7.1/02). The cut off for statistical significance was defined as $p \leq 0.05$ or $p \leq 0.01$ as necessary.

Table KIIA 5.7.1/02. Statistical Methods

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant	**If preliminary test is significant**
Bodyweight gain Terminal bodyweight Abs and rel organ weights Select FOB parameters (spontaneous motor activity, grip strength, foot splay, rectal temperature)	Bartlett Test	**One-way analysis of variance** followed by 2-sided **Dunnett's test**	**Kruskal-Wallis test** followed by 2-sided **Dunn's test**
Bodyweight	Bartlett Test	One-way analysis of variance followed by 2-sided **Dunnett's test**	Log transformed data **Bartlett Test,** if significant then **Kruskal-Wallis test** followed by 2-sided **Dunn's test;** Otherwise **ANOVA** and 2-sided **Dunnett tests** on log transformed data

C. METHODS

1. Observations

Observations for mortality and moribundity were undertaken twice daily (or daily for weekends/public holidays) for 14 days, and daily observations were made for clinical/behavioural signs and evidence of abnormal excreta or body fluids in cage trays.

2. Body weights

Animals were weighed prior to dosing on test Day 1 and on Study Days 7 and 14.

3. Food consumption and food use efficiency

Food consumption was not recorded.

4. Functional observational battery (FOB) and motor activity (MA)

For the first dosing cohort animals, neurotoxicity assessment, including a full FOB and motor activity assessments were undertaken immediately prior to dosing, and at two hours, seven days, and 14 days post-dosing. The parameters evaluated in the FOB assessment are presented below.

Inside the home cage:

- Posture
- Piloerection
- Involuntary motor movements
- Gait/coordination abnormalities
- Vocalisations
- Any 'abnormal' behaviour

During removal from the home cage and handling:

- Ease of removal
- Reaction to handling
- Eyelid position
- Lacrimation
- Muscle tone
- Salivation
- Fur staining or alopecia
- Emaciation
- Nasal discharge
- Temperature upon touching

Individual open field observations (two minutes/animal):

- Piloerection
- Posture
- Arousal
- Gait abnormalities
- Involuntary motor movements
- Stereotypic movements
- Vocalisations
- Respiration rate
- Number of rearing movements
- Urine and faeces spots

The remainder of FOB testing involved standardized or calibrated devices (only pupil size and pupil reflex were assessed in second cohort females):

- Fore- and hindlimb grip strength (strain gauge)
- Landing foot splay
- Rectal temperature
- Pupil size
- Pupillary constriction with a beam of light
- Corneal reflex (response to touching medial canthus)
- Surface righting reflex
- Flexor reflex (response to pinching toes)
- Auditory startle response
- Tail pinch response
- Body weight

Motor Activity (MA) (first cohort groups only):

Animals were tested individually for exploratory activity in a novel environment using an automated photocell recording apparatus (Imetronic, Bordeaux, France). Exploratory activity was recorded during

the first 60 minutes, with data collected at 10-minute intervals throughout the session.

In the follow-up female groups receiving 0, 20, or 35 mg/kg bw/day, a partial FOB was undertaken prior to dosing and at 2 hours post dosing - a time known from the initial study to coincide with the peak toxicological effects, after which animals were sacrificed and discarded without further examination. The analyses included only procedures listed in the home cage, removal and handling, open field observation, and pupil size and pupil reflex procedures listed below (i.e., remainder of FOB tests involved standardised or calibrated devices and motor activity tests were not undertaken in the second cohort female groups).

Technicians undertaking FOB analyses were unaware of dosing levels of the animals being examined.

5. Sacrifice and neuropathology

On Study days 15, 16, or 17, six animals/dose in the main study received *i.p*. injections of 60 mg/kg bw/day heparin sodium, and then were exsanguinated under isoflurane induced anesthesia. Simultaneously, animals were perfused via the left ventricle with an initial flush of phosphate buffer, followed by a fixative solution of 4% formaldehyde and 1% glutaraldehyde in phosphate buffer. Necropsy examination was then undertaken and confined to external tissues and macroscopic lesions in neural tissues. Replacement of animals due to inadequate perfusion and/or death during anesthesia was used as necessary. The fixed tissues in these animals were then subjected to histopathological examination.

Histopathological analyses

In the perfused animals, dissection was undertaken for: Brain, dorsal root ganglia and cervical and lumbar swellings spinal nerve roots, eyes, gasserian ganglia (bilateral), gastrocnemius muscle (unilateral), optical nerves (bilateral), peripheral nerves (sciatic, tibial, and sural nerves) (bilateral), spinal cord (cervical, thoracic and lumbar), macroscopic lesions in neural or skeletal muscle.

The collected samples were post-fixed in the fixative solution used for the perfusions (a solution of 4% formaldehyde and 1% glutaraldehyde in phosphate buffer), except the eyes and optic nerves which were fixed in Davidson's fixative.

The brain was trimmed using a metal rodent brain matrix, and eight coronal sections included parts from the major brain regions: Olfactory bulbs, cerebral cortex, caudate putamen, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, pons and medulla oblongata.

The following samples were embedded in paraffin and sectioned at 4 μm and stained by heamatoxylin and eosin staining: Brain, spinal cord (cervical, thoracic, lumbar), eyes, optic nerves, and gastrocnemius muscle.

The following samples were embedded in glycol methacrylate, sectioned at approx. 2 to 3 μm thickenss and stained using Lee's stain: Cervical and lumbar swellings of dorsal root ganglia (including dorsal and ventral root fibres), gasserian ganglia, and peripheral sciatic, tibial, and sural nerves. The left side only of the dorsal root ganglia and peripheral nerves were histologically examined.

The stained samples from control and 800 mg/kg bw/day group animals were subjected to histopathological analysis by light microscopy by an initial examiner, and all slides were reviewed by a second pathologist. As no histopathological abnormalities were found, samples from other dosing groups were not analysed.

The remaining 6/sex/dose animals in the main study were sacrificed by exsanguination under isoflurane anaesthesia, and subjected to a gross necropsy, including examination of all orifices and major body cavities. Histopathological analyses were not undertaken for these animals.

Females used in the follow-up study were sacrificed after FOB analyses and discarded without further examination.

6. Positive control data

The study report stated that procedures and data describing the effects of the positive controls carbaryl and trimethyltin in this laboratory had been previously documented in two separate reports:

1) Garcin JC (2011) Carbaryl, An experimental functional observation battery validation study in rats, Bayer CropScience, Sophia Antipolis, France (SA 09246).
2) Garcin JC (2011) Trimethyltin, An acute neurotoxicity study after a single i.p. administration in rats, Bayer CropScience, Sophia Antipolis, France (SA 09296).

Based on these studies the experimental procedure was considered to be adequate to detect clinical signs and histopathological correlates of neurotoxicity.

II. RESULTS AND DISCUSSION

A. MORTALITY AND CLINICAL SIGNS

There were two instances of premature mortality at 800 mg/kg bw/day, with a female (UT4F2486) that died during neurobehavioral testing on the day of dosing (peak period of effect) and the other female (UT4F2483) found dead on study Day 5. The study author regarded both mortalities as being test substance related. In the prematurely dead female (UT4F2486) on the day of dosing, prior to death clinical signs consisted of piloerection, low muscle tone and arousal, rapid respiration, tremors, myoclonic jerks, convulsions, dilated pupils, absence of pupil and flexor reflexes and uncoordinated or slow surface righting reflex. No deaths were recorded in other treatment and control groups.

No treatment-related clinical signs were observed at the daily observations throughout the study.

B. BODY WEIGHT

Effects on bodyweight gain were confined to 40/46% (M/F) lower mean bodyweight gains at 800 mg/kg bw/day in study week 1 relative to controls ($p \leq 0.01$ for M; $p \leq 0.05$ for F). However, group mean bodyweights at 800 mg/kg bw/day on study day 7 were within 3.5% of that for controls, and by study day 14 there were no differences in group mean bodyweight or bodyweight gain.

Group mean bodyweight gain and bodyweights were unaffected in all other test substance groups.

C FOOD CONSUMPTION

Food consumption data were not recorded.

D FUNCTIONAL OBSERVATIONAL BATTERY (FOB) AND MOTOR ACTIVITY

In-cage observations

On study Day 1 during in-cage observations, clinical signs consisted of an increased incidence of piloerection in males at 200 (7/12) and 800 mg/kg bw/day (9/12) compared to controls (4/12), while the incidence at 50 mg/kg bw/day was considered unaffected by treatment (3/12). In females, an increased incidence of piloerection was seen at 50 (6/12) and 800 (8/12) mg/kg bw/day relative to that in controls (2/12), however the incidence at 200 mg/kg bw/day was considered unaffected by treatment (3/12). There was no effect on in-cage FOB parameters in females at 20 or 35 mg/kg bw/day. In-cage FOB parameters at study Day 7 or 14 were unremarkable for all groups.

Handling observations

Treatment-related changes in handling observation parameters were confined to study day 1 only.

On study Day 1 during handling observations, increased incidences were seen in flaccid muscle tone at 800 mg/kg bw/day (5/12 for M; 8/12 for F) and 200 mg/kg bw/day (3/12 for M; 1/12 for F) compared to controls (1/12 for M; 0/12 for F). An increased incidence of coldness to the touch was seen for males (10/12) and females (9/12) at 800 mg/kg bw/day, and in males at 200 mg/kg bw/day (2/12) compared to an absence of the finding in controls (0/12 for both sexes). There was no effect on observations during handling in females at 20 or 35 mg/kg bw/day.

Additional findings during handling observations on day 1 were slight yellow-soiled fur (paw/nose) in males (1/12) and slight red soiled fur (nose or head) in females (2/12) at 800 mg/kg bw/day, and slight red soiled fur in one male (paw) at 200 mg/kg bw/day, versus an absence of these findings in controls. However, the findings were present in 1/12 control females in pre-study observation period as a slight severity of red staining of the nose, and therefore the evaluator considers these findings to be unrelated to the test substance.

Open field observations

Abnormal findings in open field observations were confined to study day 1 (Table KIIA 5.7.1/03)

Table KIIA 5.7.1/03: Open Field Observations (Day 1)

	Males (N=12)				**Females (N=12)**			
Dose (ppm)	**0**	**50**	**200**	**800**	**0**	**50**	**200**	**800**
Piloerection	4	8	12	12	4	7	11	12
Rapid respiration	1	0	9	12	0	0	6	12
Low arousal	1	1	6	11	0	0	1	8
Clonic tremors	0	0	4	8	0	0	4	10
Myoclonic jerks	0	0	1	5	0	0	0	7
Clonic convulsion	0	0	0	5	0	0	0	1
Chewing	0	0	0	5	0	0	1	4

Vocalizations	0	0	3	0	0	0	1	2
Incoordination	0	0	3	7	0	0	5	5
Waking on tiptoes (females only)					1	1	5	4
Hunched posture &/or Flatted body	3	2	8	11	0	0	3	11
Rearing (mean ±SD)	7.2 ± 2.4	7.8 ± 3.4	4.3 ± 3.6	2.7 ±2.8	12.3 ± 4.6	10.7 ± 3.3	9.9 ±4.6	2.8 ±2.8

Data excerpted from pages 87 & 88 of the study report. (Compiled by US EPA secondary reviewer)

Open field observational findings on study Day 1 in the 800 mg/kg bw/day groups reported involuntary motor movements in the form of clonic tremors (8/12 for M and 10/12 for F), myoclonic jerks (5/12 for M and 7/12 for F), gait incoordination (7/12 for M and 5/12 for F) or absence of movement (2/12 for M and 3/12 for F), and clonic chewing (5/12 for M and 4/12 for F), versus an absence of these findings in controls.

Additional findings at this dose and timepoint were increased incidences of piloerection (12/12 for both M & F, *vs* 4/12 for controls), rapid respiration (12/12 for both M & F, *vs* 1/12 and 0/12 for control M and F), low arousal (11/12 for M, 8/12 for F, *vs* 1/12 and 0/12 for control M and F), repetitive licking of lips (2/12 for M and 8/12 for F, *vs* 1/12 and 0/12 for control M and F), walking on tiptoes (4/12 for F, *vs* 1/12 for control F), and flattened or hunched posture (11/12 for both M and F, *vs* 3/12 and 0/12 for control M and F). A lower incidence of rearing was also present at this dose (group means of 2.7±2.8 incidences for M and 2.8±2.8 incidences for F, *vs* 7.2±2.4 and 12.3±4.6 for M and F controls, respectively).

At 200 mg/kg bw/day on study Day 1, similar findings to 800 mg/kg /bw animals were observed, but at lower incidences and/or severity. Incidences were observed of clonic tremors (4/12 for both M and for F) and gait incoordination (3/12 for M and 5/12 for F), and in males only, myoclonic jerks (1/12 for M) and absence of movement (3/12 for M), versus an absence of these findings in controls.

Additional findings at this dose and timepoint were increased incidences of piloerection (12/12 for M and 11/12 for F; *vs* 4/12 for M and F controls), rapid respiration (9/12 for M and 6/12 for F; *vs* 1/12 and 0/12 for control M and F, respectively), low arousal (6/12 for M and 1/12 for F; *vs* 1/12 and 0/12 for control M and F), walking on tiptoes (5/12 for F, *vs* 1/12 for control F), flattened or hunched posture (8/12 for M and 3/12 for F, *vs* 3/12 and 0/12 for control M and F), and lower rearing incidence (group mean incidence of 4.3±3.6 for M and 9.9±4.6 for F, *vs* 7.2±2.4 and 12.3±4.6 for M and F controls, respectively).

At 50 mg/kg bw/day on study day 1, abnormal treatment-related FOB findings were confined to increased incidences of piloerection in both sexes (8/12 for M and 7/12 for F, *vs* 4/12 for M and F controls). At 20 and 35 mg/kg bw/day in females, there were no test substance related FOB findings for open field observations.

V

Sensory reactivity

Abnormal findings in sensory reactivity parameters were confined to study day 1. While increased incidence of uncoordinated or slow righting reflexes were seen in males at 800 mg/kg bw/day on study Day 14 (5/12, *vs* 1/12 for controls), the study authors considered this to be unrelated to the test substance given the absence of this finding on study Day 7 (2/12, *vs* 4/12 in controls). The evaluator agrees that this specific finding at study day 14 was unrelated to the test substance.

On study Day 1 at 800 mg/kg bw/day, weak tail pinch responses (2/12 for M and 6/12 for F, no incidence in controls) were observed. Increased incidences of dilated pupils (6/12 for M and 10/12 for F, *vs* 0/12 and 1/12 for control M and F; equal responses between left and right eyes), uncoordinated or slow righting reflex (6/12 for M and 5/12 for F, *vs* 0/12 for controls, noting incidences of up to 3/12 in M and F during pre-dosing baseline observations), exaggerated flexor reflexes (up to 4/12 for M and 2/12 for F, *vs* 0/12 and 1/12 for control M and F; left and right paws), and moderate flexion responses (up to 2/12 for M and 5/12 for F, *vs* 1/12 and 0/12 for control M and F; left and right paws) were also noted.

At 200 mg/kg bw/day at study Day 1, increased incidences were observed for dilated pupils (4/12 for M and 7/12 for F, *vs* 0/12 and 1/12 for control M and F; equal responses between left and right eyes) and exaggerated flexor reflexes (3/12 for M and 2/12 for F, *vs* 0/12 and 1/12 for control M and F).

At 50 mg/kg bw/day, an increased incidence of pupil dilation was observed in females (6/12 *vs* 1/12 for control F; equal responses between left and right eyes). At 20 and 35 mg/kg bw/day in females or females, there were no test substance related sensory reactivity findings.

Grip strength, landing foot splay, rectal temperature and bodyweights

On study Day 1, statistically significant ($p \leq 0.01$) lower rectal temperatures were observed at 800 mg/kg bw/day, in males (4.1% decrease; 35.3°C *vs* 36.8 °C in controls) and females (6.7% decrease; 35.0°C *vs* 37.5 °C in controls) relative to controls, though the effect resolved by study Day 7.

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There was no effect on grip strength, foot splay, or bodyweight. A dose dependent trend for decreased landing foot splay (Table KIIA 5.7.1/03) was seen that did not reach statistical significance and was not regarded by the study authors to be test substance related. The evaluator agrees that this finding was unrelated to the test substance, taking into consideration the variability noted in control animals as well as the values for all groups when compared to pre-test values.

Table KIIA 5.7.1/03: Landing foot splay data (cm) on study day 1

	Dose (mg/kg bw/day)							
	M				**F**			
	0	**50**	**200**	**800**	**0**	**50**	**200**	**800**
	8.1±2.2	7.4±1.3	7.1±1.3	**6.8±1.0**	7.5±2.0	7.7±1.1	6.6±1.4	6.1±1.1
%ΔC	-	**-9**	**-12**	**-16**		**3**	**-12**	**-19**

'%ΔC' – denotes per cent change from control. No landing foot splay findings reached statistical significance ($p \leq 0.05$). Landing foot splay responses were not assessed in the female-only follow-up study groups at 20 and 35 mg/kg bw/day. Data excerpted from pages 116 and 117 of the study report, with the exception of per cent change from control, which was calculated by the evaluator using Microsoft Excel.

Exploratory locomotor activity

At 800 mg/kg bw/day on Day 1, spontaneous motor activity during the 60 minutes test session was statistically significantly ($p \leq 0.01$) lower in both males (50% decrease) and females (61% decrease) relative to respective controls, with severity being greatest in the first 10 minutes of the session (71/73% lower than controls for M/F, respectively).

At 200 mg/kg bw/day on Day 1, statistically significantly ($p \leq 0.01$) lower (40/47% lower in M/F) motor activity was seen in the first ten minutes but not thereafter in the observation period, with overall exploratory motor activity comparable with controls (Table KIIA 5.7.1/04). No effect on locomotor activity was seen in lower dosing groups on study Day 1, or in any groups on study Days 7 or 14.

Table KIIA 5.7.1/04: Exploratory locomotor activity on study day 1

	Dose (mg/kg bw/day)							
Minutes	**M**				**F**			
	0	**50**	**200**	**800**	**0**	**50**	**200**	**800**
Pre-test total	172 ± 87	171 ± 69	234 ± 86	189 ± 146	291 ± 176	329 ± 170	300 ± 126	265 ± 154
Day 1								
1–10	107 ± 37	120 ± 37	**64 ± 32***	**31 ± 17***	136 ± 31	111 ± 28	**72 ± 29***	**37 ± 23***
11–20	38 ± 32	43 ± 30	50 ± 50	16 ± 14	55 ± 26	41 ± 30	43 ± 35	**18 ± 19***
21–30	13 ± 17	30 ± 34	13 ± 15	18 ± 19	38 ± 45	37 ± 35	22 ± 35	8 ± 16
31–40	16 ± 30	23 ± 39	10 ± 15	6 ± 7	20 ± 29	30 ± 35	20 ± 33	16 ± 42
41–50	6 ± 8	17 ± 27	11 ± 23	10 ± 15	23 ± 27	30 ± 52	27 ± 32	20 ± 18
51–60	15 ± 25	21 ± 38	15 ±27	16 ± 16	23 ± 37	23 ± 29	14 ± 21	15 ± 24
Day 1 Total	194 ± 47	254 ± 118	163 ± 92	**97 ± 29***	293 ± 156	272 ± 154	199 ± 125	**115 ± 72***

* $p < 0.01$ vs controls.

In the follow-up study, the animals at 20 and 35 mg/kg showed no treatment-related effects in all the parameters examined.

F. GROSS PATHOLOGY AND NEUROPATHOLOGY

Necropsy and histopathology findings including those in central nervous system tissues were unremarkable. No treatment-related effects on brain weight were observed.

III. CONCLUSIONS

When BYI 02960 was administered to test rats in a single gavage dose of 0, 50, 200, or 800 mg/kg bw/day; in addition, a follow up cohort of 12 females/dose receiving 0, 20, or 35 mg/kg bw/day oral gavage doses was added.

Under the condition of the study, no gross or microscopic test substance-related morphological changes were seen in the nervous system tissues of all doses tested.

Based on the presence of increased incidences of piloerection in both sexes and dilated pupils in females at 50 mg/kg bw/day on study day 1, the study author regarded the NOAEL in the study to be 35 mg/kg bw/day, with an absence of any test substance related findings in females at this dose. As 50 mg/kg bw/day was the lowest dose tested for males, a NOAEL for this sex was not determined. However, the study author regarded the NOAEL of 35 mg/kg bw/day to be appropriate for males also. Although inclusion of a male group at this dose would have been preferable particularly given piloerection incidence was slightly higher in males than females at 50 mg/kg bw/day, there was no clear indication that males were more sensitive to this clinical signs of toxicity. The evaluator therefore considers the study author's conclusions to be appropriate with respect to general

observations and clinical signs of toxicity.

The NOAEL in this acute neurotoxicity study is 35 mg/kg bw/day, based on the presence of increased incidences of piloerection in both sexes (8/12 for M and 7/12 for F, vs 4/12 for both M and F controls) and increased incidence of pupil dilation (6/12, vs 1/12 for controls) in females at 50 mg/kg bw/day on study day 1.

This study is fully reliable (acceptable/guideline) and satisfies the data requirements for an acute neurotoxicity study (OPPTS 870.6200; OECD 424).

Primary review: Australia
Secondary review: US EPA & PMRA

Report: Garcin JC (2011) BYI 02960 - A 90-day neurotoxicity study in the rat by dietary administration. Bayer S.A.S., Bayer CropScience, 355, rue Dostoievski, BP 153, 06903 Sophia Antipolis Cedex. France. 28 June 2011. MRID 48844139. PMRA 2239524. Study No. SA 09283. Study sponsor: Bayer AG, Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N°TXRVP022. Unpublished.

Guidelines: U.S. EPA OPPTS 870.6200 (1998)
OECD 424 (1997)
MAFF Japan Nousan 8147 (2000) No. 12

Deviations: Animals were not observed for clinical signs on the final day of sacrifice. These deviations were considered by the evaluator not to have affected the overall reliability of the study findings.

GLP: Yes Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:

In a subchronic neurotoxicity feeding study (MRID 48844139), seven-to-eight week old Wistar Rj:WI (IOPS HAN) rats in groups of 12/sex/dose received BYI 02960 (96.2%; 2009-000239) in the diet at concentrations of 0, 100, 500, and 2500 ppm (0/0, 5.7/6.9, 29.4/34.8, or 143/173 mg/kg bw/day for M/F) for 90 days. The following parameters were evaluated: bodyweights, bodyweight gain, food consumption, clinical signs, detailed physical examination, ophthalmology, and gross pathology. A neurobehavioral test battery consisting of motor activity and functional observational battery (FOB) assessments was conducted on all animals prior to BYI 02960 dosing (baseline) and during study weeks 2, 4, 8, and 13 or 14, depending on sacrifice schedule. On test days 92 and 93, 6 rats/sex/group were perfused *in situ* with fixative. The peripheral and central nervous systems and selected muscle tissues from perfused control and high dose groups were prepared for histological evaluation.

Under the conditions of the study, there were no test substance-related effects on the following parameters: mortality, ophthalmology, neurobehavioral parameters (FOB and motor activity), or gross and microscopic morphology of the nervous system in either male or female rats of any dose groups.

There was a decrease in absolute body weight in males and females at 2500 ppm reaching 9% at week 1 for females and at weeks 4 and 8 for males. The magnitude of reduced body weight did not persist or approach the level of being adverse. Associated mean bodyweight gains were also decreased for both sexes at 2500 ppm relative to controls from study week 1 onwards.

There were decreases in food consumptions in 2500 ppm males and female (↓18/29% (M/F) $p \leq 0.01$) over study week 1, which partially recovered over the study to reach a 9/4% (M/F) lower consumption relative to controls on study week 13.

In this 90-day neurotoxicity study with dietary administration, flupyradifurone did not produce neurotoxicity at any dose levels tested. The no-observed-adverse-effect level (NOAEL) was 500 ppm (29.4/34.8 mg/kg bw/day; M/F); LOAEL was 2500 ppm (143/147 mg/kg bw/day; M/F) based on decreased food consumption with associated slight decrease in absolute body weight.

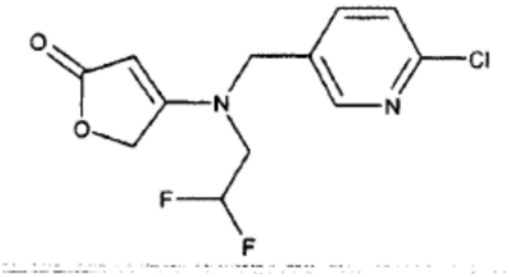
This study is classified as fully reliable (acceptable/guideline) and meets the guideline requirements

of subchronic neurotoxicity study (U.S. EPA OPPTS 870.6200; OECD 424; and MAFF Japan Nousan 8147).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   4-[6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one



Lot/Batch #:	2009-000239
Purity:	96.2% (1H-NMR; 16 Jan 2009)
Description:	Beige powder
CAS #:	951659-40-8
Stability of test compound:	The test substance was stored in an air-tight, light-resistant container at room temperature (25±5ºC). Stated to be stable for 2 years if stored as recommended. Stability analysis of admixed feed was undertaken in study No. SA 07294, where storage at room temperature for 11 days led to degradation of up to 6% (93.6% at day 0 down to 87%) for 100 ppm batches of feed and 0% (93.5% at day 0 up to 95%) by 11 days for 2500 ppm batches of feed. Prior freezing at -20°C for up to 92 days before being left at room temperature for 10 days did not result in additional degradation greater than storage for 10 days at room temperature alone.

2. Vehicle: Prepared diet (dietary admixture)
3. Test animals

Species:	Rat
Strain:	Wistar Rj:WI (IOPS HAN)
Age at dosing:	7 to 8 weeks old
Weight at dosing:	232 to 339 g for males; 191 to 230 g for females
Source:	R. Janvier, Le Genest St Isle, France.
Acclimation period:	19 days
Diet:	A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) *ad libitum*, except at designated examination time periods.
Water:	Filtered and softened tap water, *ad libitum*, except at designated examination time periods.

Housing: Individually in suspended, stainless steel and wire mesh cages.

4. Environmental conditions

Temperature: 20 to 24°C
Humidity: 40 to 70%
Air changes: 10 to 15/hr
Photoperiod: Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion

2 November 2009 to 16 June 2010

2. Animal assignment and treatment

Four groups of 12 animals/sex/dose received BYI 02960 in feed daily for 90 days at dietary concentrations of 0, 100, 500, and 2500 ppm (Table KIIA 5.7.4/01 and Table KIIA 5.7.4/02). The dosing regimen was based on the results of the 90-day dietary study in rats with the test substance (Odin-Feurtet, 2009; Study No SA 07294) where the study authors set a NOAEL of 500 ppm, based on a LOAEL of 2500 ppm for reduced bodyweight, bodyweight gain and food consumption, and changes in platelet count, bilirubin, glucose, cholesterol and triglycerides and altered histopathology findings in liver and thyroid.

Animals were assigned to dose groups by computerised, stratified randomisation so that all animal starting bodyweights were within ±20% of the group mean bodyweight within a sex. A negative control group received untreated diet.

Table KIIA 5.7.4/01: Study Design/dosing regime

Conc. In diet (ppm)	No./sex group	Mean intakes[b] (mg/kg bw/day)		Animal IDs	
		M	F	M	F
0	12	0	0	TT1M4861 to 4872	TT1F4873 to 4884
100	12	5.7±1.0	6.9±0.8	TT2M4885 to 4896	TT2F4897 to 4908
500	12	29.4±4.9	34.8±4.3	TT3M4909 to 4920	TT3F4921 to 4932
2500	12	143.0±21.1	173.0±16.8	TT4M4933 to 4944	TT4F4945 to 4956

[a] Unadjusted for purity or potential degradation.
Data excerpted from pages 13 and 18 of the study report, except for standard deviations which were calculated by the evaluator using Microsoft Excel and data on page 65 of the study report.

Table KIIA 5.7.4/02: Test substance intake

Study week	achieved weekly mean test substance intake (mg/kg bw/day)					
	M, nominal dose (ppm)			F, nominal dose (ppm)		
	100	500	2500	100	500	2500
1	7.8	39.4	173	8.7	43.0	178
2	7.0	36.2	180	7.9	40.7	205
3	6.5	34.4	165	7.6	39.4	201
4	6.0	32.2	159	7.2	37.5	188
5	5.9	30.8	152	7.0	36.1	181
6	5.5	29.5	146	7.0	34.6	173
7	5.3	26.6	132	6.8	33.6	169
8	4.9	26.1	135	6.3	31.6	161
9	5.2	26.6	132	6.3	32.9	165

10	5.0	26.2	127	6.4	31.8	161
11	4.8	24.7	120	6.2	30.2	157
12	4.9	24.9	120	6.2	30.5	154
13	4.7	24.3	118	6.2	30.0	156
Mean± SD	**5.7±1.0**	**29.4±4.9**	**143.0±21.1**	**6.9±0.8**	**34.8±4.3**	**173.0±16.8**

Unadjusted for compound purity or degradation. Means provided by the study authors (p65 of study report), and SD calculated by the evaluator using weekly means only and the Standard deviation function in Microsoft Excel (*i.e.*, Standard deviations are an underestimate as they exclude standard deviations within each week).

3. Diet preparation and analysis

The test substance was incorporated into the diet by dry mixing to provide the required dietary concentrations. Two batches of diet were prepared for each concentration, with the first batch used up to study week 8 and the second used thereafter until study termination. Batches of prepared diet were stored at room temperature when not in use. Additionally, samples of each concentration of feed were stored at -20°C for potential future analysis.

Homogeneity/concentration analysis was undertaken for the first 100 and 2500 ppm batches, and concentration analysis was undertaken on 500 ppm batches and the second batches of 100 and 2500 ppm. For homogeneity analysis, three samples were taken from three levels in feed piles, surface (H), middle (M), and bottom (B), giving a total of nine samples per batch of prepared diet. For concentration analysis of the first 500 ppm batch, single samples were taken at three levels (surface, middle and bottom) following a descending diagonal, pooled and then analysed.

For concentration analysis of the second batches at 100, 500 and 2500 ppm, two samples were taken from unspecified locations, of which one was analysed and the other was stored for potential follow up analysis. Achieved homogeneity and concentration ranged between 91 and 104% of the nominal, and were regarded by the study authors to be within the acceptable range of 85 to 115% of nominal concentrations (Table KIIA 5.7.4/03).

Stability analysis was not undertaken, with the study authors instead citing the 90-day dietary toxicity study in rats with BYI02960 by Odin-Feurtet (2012; Report No. SA 07294), which was considered representative of the stability of the test substance in dietary admixture. The evaluator considers that the results from the stability study cited to be applicable to this study in this case.

Table KIIA 5.7.4/03: Homogeneity and concentration data in prepared feed batches.

Nominal conc. in diet (ppm)	Date of analysis (date of formulation)	Layer obtained from batch (H, M, B)	Achieved concentration (ppm)	% of nominal (±SD)
0				
	19 Nov 2009	not reported	<VL	-
	12 Jan 2010		<VL	-
100				
F1bis	19 Nov 2009	H1	97.6	*98*
		H2	99.2	*99*
		H3	97.3	*97*
		M1	95.2	*95*
		M2	98.9	*99*
		M3	99.0	*99*
		B1	98.6	*99*
		B2	99.9	*100*
		B3	98.1	*98*
		H1 to B3 Mean	**98.2±1.4**	**98.2±1.5**
F2	12 Jan 2010	not reported	91.6	**92**
500				
F1bis	19 Nov 2009	not reported	499	**100**
F2	12 Jan 2010	not reported	465	**93**
2500				
F1bis	19 Nov 2009	H1	2486	*99*
		H2	2550	*102*
		H3	2542	*102*
		M1	2560	*102*
		M2	2588	*104*
		M3	2564	*103*
		B1	2552	*102*
		B2	2562	*102*
		B3	2460	*98*
		H1 to B3 Mean	**2540.4±40.8**	**102±1.9**
F2	12 Jan 2010	not reported	2271	**91**

Prior to use, batch F1 load 1 was found to have been formulated within canine diet instead of rodent diet, and replaced with batch F1bis. 'H' denotes highest layer of batch, 'M' denotes middle layer, and 'B' denotes bottom layer. '<VL' denotes below validation limit (<25 ppm). Date of analysis 14 Feb 2008 (for F1bis), Feb 25 2008 (F2b load 1), or 27 March 2008 (for F2). Data excerpted from pages 614 and 615 of the study report, except for standard deviations, which were calculated by the evaluator using Microsoft Excel.

4. Statistics:

Analyses were undertaken on bodyweights, organ weights, and selected FOB parameters, bodyweight change and mean food consumption per day using the Path/Tox System V4.2.2. (Module Enhanced Statistics). Statistical significance was defined as group mean differences $p \leq 0.05$. A step-wise process was used where a Bartlett Test was used as the preliminary test. Statistical methods used in this study are presented in Table KIIA 5.7.4/04.

Table KIIA 5.7.4/04: Statistics used in data analyses

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant (p>0.05)	**If preliminary test is significant (p≤0.05)**
Bodyweight change Terminal bodyweight Spontaneous motor activity, fore- and hindlimb grip strength, landing foot splay and rectal temperature parameters. Organ weights (absolute & relative)	**Bartlett Test**	**One-way analysis of variance**, followed by 2-sided **Dunnett's test if significant**	**Kruskal-Wallis test** followed by 2-sided **Dunn test if significant**
Bodyweight Mean food consumption per day	**Bartlett Test**	**One-way analysis of variance** followed by 2-sided **Dunnett's test if significant**	Log transformed data **Bartlett Test,** if significant then **Kruskal-Wallis test** followed by 2-sided **Dunn's test (if significant);** Otherwise **ANOVA** followed by **Dunnett tests** on log transformed data if significant.

C. METHODS

1. Observations, Functional Observation Battery and motor activity

During the acclimation phase, all animals were observed daily for mortality and morbidity, and were subjected once to a physical and ophthalmological examination. During the dosing period, all animals were observed for mortality twice daily (once daily on weekends and public holiday) and for abnormal clinical signs at least daily, and a detailed physical examination was undertaken on a weekly basis. The nature, onset, severity, duration and recovery of clinical signs were recorded, and cages and cage trays were inspected daily for evidence of ill health.

Additionally, during acclimation and on study weeks 2, 4, 8, and 13 or 14, all surviving animals were subjected to Functional Observation Battery and motor activity assessment by animal technicians blinded to the animals' dosing regimen.

The parameters evaluated in the FOB assessment are presented below.

Home cage observations:
Posture, piloerection, involuntary motor movements, gait abnormalities, vocalisations, abnormal behavior.

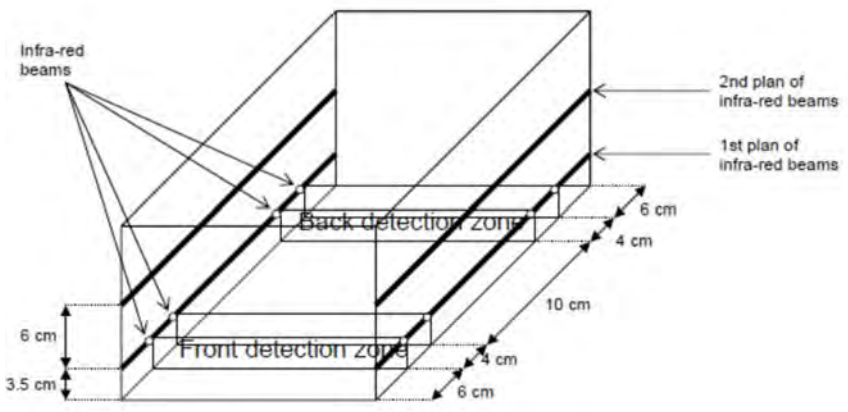
Handling observations:
Ease to remove from cage, reaction to being handled, muscle tone, eyelid, lacrimation, salivation, nasal discharge, staining or any other signs such as alopecia, emaciation, temperature upon touching.

Open-field observations (individually for two minutes):
Piloerection, respiration, arousal, gait abnormalities, posture, involuntary motor movements, stereotypic movements, vocalisations and number of rearings, urine and faeces spots

Reflex and physiologic observations/measurements:
- Pupil size
- Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary constriction by focusing a narrow beam of light in the eyes).
- Surface righting reflex (by putting the animal on its back and evaluating its ability/rapidity to reassume a normal standing position).
- Corneal reflex (by touching the medial canthus with a fine object and observing the quick and complete closure of the eyelids).
- Flexor reflex (by pinching the toes and evaluating the presence/strength of the flexor response of each hindlimb).
- Auditory startle response (by evaluating the animal response to an auditory stimulus).
- Tail pinch response (by pinching the tail with a forceps and evaluating the animal reaction).
- Grip strength: the fore- and hindlimb grip strength of animals was measured quantitatively using a grip strength apparatus equipped with one pull and one push strain gauge (Bioseb, Chaville, France).
- Landing foot splay: the animal was dropped from approximately 30 cm above the surface and hindlimb foot splay was marked, measured and recorded.
- Body weight
- Rectal temperature

Exploratory locomotor activity (MA):
Rats were tested individually in automated photocell apparatus (Imetronic, Bordeaux, France) that recorded spontaneous exploratory locomotor activity in the novel environment of the apparatus over 65 minutes with data collected at 10-minute intervals throughout this period. A schema of the automated photocell apparatus provided by the study authors is reproduced below (Figure KIIA 5.7.4/A).

Figure KIIA 5.7.4/A: Diagram of the automated photocell apparatus



The figure above was excerpted from page 23 of the study report.

2. Body weights

Animals were weighed three times during acclimation, once on the day of administration and at least weekly thereafter, and once immediately prior to scheduled sacrifice.

3. Food consumption, food efficiency, and daily intake

Food consumption was recorded weekly, and any instances of spillage were noted. This data together with bodyweights were used to estimate mean daily feed and test substance intakes.

The mean achieved test substance intake was defined using the following formula:

$$\text{intake (mg/kg bw/day)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean bodyweight at end of selected week interval (g)}}$$

Food efficiency was not evaluated.

4. Ophthalmoscopic examination

All animals were subjected to ophthalmological examination by indirect ophthalmoscope following instillation of the atropinic agent Mydriaticum™ (Merck Sharp and Dohme) during acclimation and at week 13.

5 Sacrifice and neuropathology

At termination, 6/sex/group were intraperitoneally injected with 60 mg/kg bw heparin sodium, anesthetised by isoflurane inhalation, exsanguinated, and subjected to whole body *in situ* perfusion. Perfusion was undertaken through the left ventricle with an initial phosphate buffer flush followed by injection of a fixative solution of 4% formaldehyde and 1% glutaraldehyde in phosphate buffer. Necropsy was confined to external and neuropathology examinations, and only macroscopic lesions from nervous system tissue or skeletal muscle were collected if present for further examination. Brain weights were recorded post-fixation whereas no other organs were weighed. The remaining (up to six) animals in each group were exsanguinated under isoflurane inhalation anesthesia and subjected to gross necropsy of external surfaces, all orifices and all major body cavities, and macroscopic lesions were recorded but not sampled.

Neuropathology examinations proceeded by dissecting the following tissues from perfused

animals, with the exception of the eyes and optic nerve, and post-fixing the dissected tissues in fixative solution of 4% formaldehyde and 1% glutaraldehyde in phosphate buffer.

- Brain
- Dorsal root ganglia and their spinal nerve roots from the cervical and lumbar swellings (bilateral)
- Eyes (bilateral)
- Gasserian ganglia (bilateral)
- Gastrocnemius muscle (unilateral)
- Optic nerves (bilateral)
- Peripheral nerves (sciatic, tibial and sural nerves) (bilateral)
- Spinal cord (cervical, thoracic and lumbar)
- Macroscopic lesions in neural tissue or skeletal muscle

The tissue selection procedures are described below (Table KIIA 5.7.4/05).

The brain was dissected using a rodent brain matrix to guide the vertical sections, which were based on anatomical landmarks (RBM coronal adult rat L5007M, Oxford Instruments SARL, Saclay, France). The resulting eight coronal sections were representative of the following major brain regions: olfactory bulbs, cerebral cortex, caudate putamen, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, pons and medulla oblongata.

Table KIIA 5.7.4/05: Tissue selection and section orientation

Tissue	Sectioning orientation
Brain (levels 1 to 8)	Coronal
Spinal cord, cervical	Cross and longitudinal
Spinal cord, thoracic	Cross and longitudinal
Spinal cord, lumbar	Cross and longitudinal
Eye (bilateral)	Cross
Optic nerve (bilateral)	Longitudinal
Gastrocnemius muscle (unilateral)	Cross and longitudinal
Dorsal root ganglia and spinal nerve roots (bilateral from the cervical and lumbar swellings)*	Longitudinal
Gasserian ganglia (bilateral)	Longitudinal
Sciatic nerve (bilateral)*	Cross and longitudinal
Tibial nerve (bilateral)*	Cross and longitudinal
Sural nerve (bilateral)*	Cross and longitudinal

* Only slides from left side prepared. Table excerpted from study page 26.

Brain, spinal cord (cervical, thoracic, lumbar), eyes, optic nerves and gastrocnemius muscle were embedded in paraffin wax, sectioned at approximately 4 µm, and stained with H&E.

Dorsal root ganglia (including dorsal and ventral root fibres) from the cervical and lumbar swellings, gasserian ganglia, and peripheral nerves (sciatic, tibial and sural) were embedded in glycol methacrylate, sectioned at approximately 2 to 3 µm and stained with Lee's stain. For the dorsal root ganglia and peripheral nerves, only tissues from the left side were evaluated.

Prepared tissues from control and 2500 ppm group animals were processed to slides and subjected to histopathological analysis.

6 Positive control data

The study report stated that the adequacy of training of technical personnel and inter-observer reliability have been documented in two separate reports:

1) Garcin JC (2011) Carbaryl, An experimental functional observation battery validation study in rats, Bayer CropScience, Sophia Antipolis, France (SA 09246).
2) Garcin JC (2011) Trimethyltin, An acute neurotoxicity study after a single i.p. administration in rats, Bayer CropScience, Sophia Antipolis, France (SA 09296).

Based on these studies, the experimental procedure was considered to be adequate to detect clinical signs and histopathological correlates of neurotoxicity.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

The study authors stated that no treatment-related clinical signs of toxicity were observed for any dietary concentration in either sex. The evaluator notes that increased salivation was seen in 2/12 females at 100 ppm, and 1/12 females and 2/12 males at 2500 ppm, but not in controls. The very low frequency of the effect without dose relationship suggests that it is likely incidental to treatment.

Ophthalmology findings were unremarkable.

2. Mortality

There were no instances of unscheduled mortality.

B. BODYWEIGHT AND BODYWEIGHT GAIN

There was a decrease in absolute body weight in males and females at 2500 ppm reaching 9% at week 1 for females and at weeks 4 and 8 for males, and a slight decrease continued till the end of the study (Table KIIA 5.7.4/06). However, the decrease in absolute body weight did not reach the magnitude of being adverse. Bodyweight gains were either absent (females in week 1 only) or lower relative to controls (both sexes) from study week 1 onwards, resulting in terminal bodyweights on study week 13 being 7/6% lower for M/F relative to controls, and overall body weight gain 15/21% lower for M/F relative to controls during the treatment period. No treatment-related changes in body weight or body weight gain were observed in 100 or 500 ppm animals.

Table KIIA 5.7.4/06: Body weights and body weight gains (g)

	Dose (ppm)							
	M				F			
	0	100	500	2500	0	100	500	2500

Initial BW (Day 1) (%C)	**296±26**	**296±20 0**	**297±22 0**	**294±18 -1**	**205±11**	**211±10 +3**	**210±10 +2**	**208±10 +1**
BW Week 1 (Day 8) (%C)	342±31	343±23 **0**	341±26 **0**	317±20 **-7**	227±12	231±15 **+2**	229±16 **+1**	207±12 **-9****
BW Week 4 (Day 29) (%C)	429±43	432±31 **+1**	433±33 **+1**	392±39 **-9***	260±15	267±16 **+3**	269±19 **+3**	246±11 **-5**
BW Week 8 (Day 57) (%C)	505±50	500±44 **-1**	512±38 **+1**	460±53 **-9**	289±20	291±15 **+1**	296±22 **+2**	269±16 **-7***
Final BW Week 13 (Day 92) (%C)	**563±55**	**561±50 0**	**569±39 +1**	**521±59 -7**	**309±24**	**313±23 +1**	**317±24 +3**	**290±19 -6**
BWG Weeks 1-4 (Days 1 to 29) (%C)	134±28	135±20 **+1**	136±17 **+1**	99±32 **-26****	55±9	56±8 **+2**	60±10 **+9**	39±6 **-29****
BWG Weeks 1-8 (Days 1 to 57) (%C)	209±35	204±34 **-2**	214±27 **+2**	166±47 **-21***	83±15	80±9 **-4**	86±15 **+4**	61±11 **-27****
Overall BWG (Days 1 to 92) (%C)	**268**±43	**265**±42 **-1**	**272**±29 **+1**	**227**±54 **-15**	**104**±18	**103**±17 **-1**	**107**±17 **+3**	**82**±16 **-21****

'%C' – denotes per cent change from control. * Statistically significant at the p≤0.05 or ** p≤0.01 level. Data excerpted from pages 28, 45-48, and 55-58 of the study report.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

The lower bodyweight gain at 2500 ppm relative to controls was at least partially accounted for by decreased absolute food consumption during study week 1 (18/29% (M/F) lower than controls). Food consumption rates partially recovered over the study to reach a 9/4% (M/F) lower consumption relative to controls on study week 13. No treatment-related food consumption differences were noted in 100 or 500 ppm animals (Table KIIA 5.7.4/07).

Table KIIA 5.7.4/07: Mean food consumption/day (g)

	Dose (ppm)							
	M				**F**			
	0	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
Initial FC Week 1 (Day 8) (%C)	26.8	26.9 0	26.9 0	21.9 **-18****	20.8	20.0 **-4**	**19.7 -5**	14.7 **-29****
FC Week 4 (Day 29) (%C)	25.9	25.9 0	27.9 **+8**	25.0 **-3**	19.3	19.1 **-1**	20.2 **+5**	18.5 **-4**
FC Week 8 (Day57) (%C)	26.1	24.3 **-7**	26.7 **+2**	24.9 **-5**	18.8	18.3 **-3**	18.7 **-1**	17.3 **-8**
Final FC Week 13 (Day 92) (%C)	**26.8**	**26.6 -1**	**27.6 +3**	**24.5 -9**	**18.8**	**19.3 +3**	**19.0 +1**	**18.1 -4**

'%C' – denotes per cent change from control. ** Statistically significant at the p≤0.01 level.
Data excerpted from page 29 of the study report.

D. FUNCTIONAL OBSERVATIONAL BATTERY AND MOTOR ACTIVITY

FOB and activity findings were unremarkable. Specifically, there were no test substance-related effects for either sex on home cage, during handling, or open-field observations, or sensory activity, grip strength, foot splay, rectal temperature or spontaneous motor activity parameters.

A trend toward increased landing foot splay was noted in males at week 8, with mean±SD values of 8.2±1.9, 9.0±2.3, 10.6±2.7, and 10.5±3.2 cm, and at week 14, with mean±SD values of 8.3±1.8, 9.6±2.2, 10.2±2.7, and 10.9±2.9 cm in the 0, 100, 500 and 2500 ppm dose groups, respectively. However, none of the mean values in the treated groups reach statistical significance when compared to control values, the dose response was weak, and no other treatment-related neurological effects were noted in this study. Therefore, this apparent dose-related increase is not considered to be an effect of treatment.

E. PATHOLOGY FINDINGS

1 Gross pathology

Changes at gross necropsy were confined to hepatomegaly in 4/6 females at 2500 ppm. The finding was also present in one female at 500 ppm, and in one male each at 500 and 2500 ppm, but was absent from concurrent controls (Table KIIA 5.7.4/08). Histopathological examination of the liver was not undertaken in this case, and therefore the basis for this finding could not be determined. The study authors noted that the finding was present in the 90-day dietary study in rats (Odin-Feurtet, 2012; Report No. SA 07294), which the evaluator notes was confined to 4/10 males and 1/10 females at 2500 ppm at terminal sacrifice. In Odin-Feurtet (2012), histopathological examination of the liver found diffuse centrilobular hepatocellular hypertrophy of minimal to slight severity in all 10 males and 3/10 females at 2500 ppm, the highest dose tested.

Table KIIA 5.7.4/08: Hepatomegaly incidence at terminal necropsy

[N=12/sex/dose]	**Dose (ppm)**							
	M				**F**			
	0	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
No. affected	0	0	**1**	**1**	0	0	**1**	**4**
% incidence	-	-	***8***	***8***	-	-	***8***	***33***

Data excerpted from page 166 of the study report, with the exception of per cent incidence, which was calculated by the evaluator using Microsoft Excel.

2. Neuropathology

Neuropathological findings were unremarkable. While a statistically significant ($p \leq 0.01$) increase in relative brain weights was recorded in 2500 ppm females (+13% vs controls), this effect was considered secondary to decreased body weight gain and not a specific neurotoxic effect. A non-statistically significant increase in relative brain weight (+12% versus controls) was also observed in 2500 ppm males, which was also considered secondary to decreased body weight gain. The absolute brain weights were comparable among all the test groups, and no dose-related changes were observed. Histological lesions noted in nervous and muscular tissue occurred in single animals and were not related to treatment.

III. CONCLUSION

Under the conditions of the study, there were no test substance-related effects on the following parameters: mortality, ophthalmology, neurobehavioral parameters (FOB and motor activity), or gross and microscopic morphology of the nervous system in either male or female rats of any dose groups.

There was a decrease in absolute body weight in males and females at 2500 ppm reaching 9% at week 1 for females and at weeks 4 and 8 for males. The magnitude of reduced body weight did not persist or approach the level of being adverse. Associated mean bodyweight gains were also decreased for both sexes at 2500 ppm relative to controls from study week 1 onwards.

There were decreases in food consumptions in 2500 ppm males and female (↓18/29% (M/F) $p \leq 0.01$) over study week 1, which partially recovered over the study to reach a 9/4% (M/F) lower consumption relative to controls on study week 13.

In this 90-day neurotoxicity study with dietary administration, flupyradifurone did not produce neurotoxicity at any dose levels tested. The no-observed-adverse-effect level (NOAEL) was 500 ppm

(29.4/34.8 mg/kg bw/day; M/F); LOAEL was 2500 ppm (143/147 mg/kg bw/day; M/F) based on decreased food consumption with associated slight decrease in absolute body weight.

This study is classified as fully reliable (acceptable/guideline) and meets the guideline requirements of subchronic neurotoxicity study (U.S. EPA OPPTS 870.6200; OECD 424; and MAFF Japan Nousan 8147).

Primary review: Australia
Secondary review: US EPA & PMRA

Report: Gilmore R (2012) A Developmental Neurotoxicity Study with Technical Grade BYI 02960 in Wistar Rats. Xenometrics, LLC, Stilwell, Kansas. Laboratory Study No. 11-D72-UW. July. MRID 48844140. PMRA 2239537. Study Sponsor: Bayer AG, Bayer CropScience, Monheim, Germany. Unpublished. 9 July 2012. Bayer CropScience File No. M-434203-01-1.

Guidelines: OPPTS 870.6300 (1998)
OECD 426 (2012)

Deviations: Exposure extended through Day 21 of lactation/postnatal development, and brain morphometry and micropathology used 21-day-old (acceptable based on OECD 426), rather than 11-day-old (as outlined in OPPTS 870.6300), offspring (10/sex/dose level).

Animal No. 0111-12 was accidentally not tested for retention seven days after the water maze task; Animal No. 0103-05 was accidentally not tested for pupil constriction on PND 21; 19 rather than 20 litters at 1200 ppm had whole brain collection and non-perfused weights collected on PND 21; Rather than 10 females and 20 litters, only 9 females and 19 litters at 1200 ppm had relative brain weights recorded on PND 75 as a bodyweight was accidentally not recorded for animal No. 3119-06; Retention on PND 30 of passive avoidance task for 1200 ppm animals was accidentally recorded in 14 rather than 15 to 16 animals in other groups; Rather than 20 or more litters/dose, only 19 litters/dose were subjected to passive avoidance and water maze assessment testing.

Historical control data or positive control data showing the sensitivity of the experimental system was cited but not provided and is not accessible. Only negative control data was provided.

The study authors stated that none of these deviations affected the reliability of the study data, providing reasons for each case of deviation. Although OECD 426 (2007) recommends provision of historical control data to demonstrate the sensitivity of the system to known neurotoxicants, especially in the case of negative results, the evaluator agreed with the study author's conclusion that the study was reliable.

GLP: Yes Signed statements of GLP, Quality Assurance, and Data Confidentiality were presented in the report.

Executive summary:

In a developmental neurotoxicity study (MRID 48844140), groups of time-mated 12-week old female Wistar Han CRL:WI (HAN) (30/dose group) received acetone-solvated BYI 02960 (96.2%; 2009-000239) in the diet at concentrations of 0, 120, 500, or 1200 ppm (0, 10.3, 42.4, or 102 mg/kg bw/day) from gestation day (GD) 6 to lactation day (LD) 21. For dams, the following parameters were evaluated: bodyweight, bodyweight gain, food consumption, mortality, clinical signs, detailed physical examination, FOB examination (GD 13 and 20 and LD 11 and 21), and reproductive outcomes. For pups, neurobehavioral evaluations including learning tasks (passive avoidance and the water maze test), motor activities, and FOB parameters were evaluated throughout the post-natal period. Additional pup parameters evaluated included bodyweight, food consumption, ophthalmology, sexual maturation, brain weights, gross necropsy, and neural and muscle tissue histopathological and morphometric analyses.

Under the conditions of this study, for dams, there were no test substance-related effects on maternal clinical signs, FOB parameters, mortality, or reproductive outcomes. For offspring, there were no test

substance related effects on clinical signs, mortality, acquisition and retention parameters for the passive avoidance and water maze tasks, sexual maturation, surface righting, pupil reflex or other ophthalmology parameters, necropsy findings, organ weights, macroscopic or histological brain morphometry, or brain or peripheral nerve histopathology.

During gestation, a decrease in body weight was seen (↓7%) in 1200 ppm dams and associated decreases in body weight gain were present. At lactation period a decrease of (4% to 6 %) in body weight was also found in1200 ppm dams. A slight decrease in food consumption was found in the dames at various times during the gestation and lactation periods (ranging from 1% to 8%). However, these changes were slight and not considered as adverse.

For pups, a non-statistically significant increase in both motor and locomotor activity was seen in males at 1200 ppm on PND 13 relative to concurrent controls. On PND 60, startle amplitude for all 50 trials was statistically significantly ($p \leq 0.05$) increased by approx. 2-fold in females at 1200 ppm relative to controls.

There was no evidence of treatment-related changes in brain morphometry or peripheral nerve histopathology.

Under the conditions of this study, the maternal NOAEL was established at 1200 ppm (102 mg/kg/day) based on no adverse effects were found in the dams at the highest dose tested.

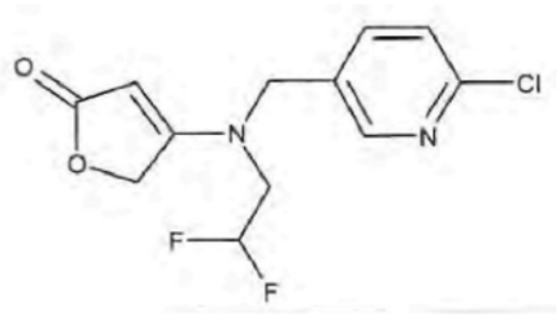
The offspring NOAEL was 500 ppm (42.4 mg/kg/day); LOAEL was 1200 ppm (102 mg/kg/day) based on statistically significant persistent increased in startle response amplitude.

This study is reliable (acceptable/guideline) and satisfies the requirements for a developmental neurotoxicity study in rats **(**OPPTS 870.6300; OECD 426).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
[4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one]



Lot/Batch #:	2009-000239
Purity:	96.2% (^{1}H-NMR; 14 Jan 2011)
Description:	Beige powder
Stability of test compound:	Analysis of stability and homogeneity of the test substance in admixed diet was not undertaken, with the study authors instead citing Moore (2010). The test substance was stated to be stable for two years from time of analysis if stored at 20±10ºC.

2. Vehicle: Acetone vehicle (concentration not reported) and incorporated into diet

3. Test animals

Species:	Rat
Strain:	Wistar Han CRL:WI (HAN)
Age at dosing:	Approx. 15 (males) and 12 (females) weeks old
Weight at dosing:	Approx. 176–225 g for females
Source:	Charles River Laboratories, Inc., Raleigh, NC
Acclimation period:	6 days
Diet:	Purina Mills Certified Rodent Diet 5002, *ad libitum*, during acclimation, except during neurobehavioural testing
Water:	Tap water, *ad libitum*, except during neurobehavioural testing
Housing:	Individually housed in wire mesh cages (66.5 and 152.0 square inches) over Deotized Animal Cage Board changed at least three times weekly, except during mating where one male and female co-habited per cage. During gestation and lactation, animals were housed individually in polycarbonate cages (162.0 square inches) with Bed-O-Cob bedding changed at least twice weekly. Cages were suspended in racks that were replaced by clean racks every two weeks.

4. Environmental conditions

Temperature:	18 to 26°C
Humidity:	30 to 70%
Air changes:	12.85 to 14.23 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion (in-life phase):
   05 July 2011 to 21 October 2011

2. Animal assignment and treatment

   The objective of this study was to investigate the potential for BYI 02960 to produce functional and morphological effects on the nervous system of offspring from dietary exposure during gestation and lactation.

   Upon receipt, animals were subjected to a six day acclimation period, immediately after which a veterinarian cleared only healthy animals for inclusion into the study.

   Approximately 120 nulliparous and non-pregnant female Wistar rats (CRL:WI, HAN) with bodyweights within 20% of the mean weight were co-housed with one male each over a period of five consecutive days in a single cage per mating pair. Each morning females and cages were examined for vaginal plug presence and vaginal smears were obtained and analysed for the presence of sperm. If the smear was positive, the day was termed GD 0, and the presumed pregnant female was removed and housed individually in a plastic cage. Females that did not present with evidence of mating after five days were sacrificed by CO_2 asphyxiation without further examination. Males allocated to breeding cages were selected at random and without bodyweight requirements. Females were sequentially (randomly) assigned to the control or test substance groups after insemination.

   From GD 6 onwards, dams in groups of 30/dose received diet containing 0, 120, 500, or 1200 ppm BYI 02960, with the test substance pre-solvated in acetone vehicle prior to incorporation into the diet (Table KIIA 5.7.5/01). The negative control group received diet containing acetone vehicle only. Provision of the test substance diets continued for dams and offspring up until lactation day (LD) 21, when all animals received diet free of the test substance and vehicle. Adjustment of test substance concentrations (reduced by 50%) were made during lactation weeks 1 to 3 to accommodate for their corresponding increases in feed intake during this period, and thereby reduce differences in achieved test substance intakes across the whole treatment period.

   Each dam was evaluated daily for evidence of delivery from GD 20 onwards, and the event was designated LD 0 for dams and PND 0 for pups. Litter size and pup "status" at birth were recorded. Litters and dams with fewer than three pups per sex or less than seven pups in total were sacrificed without necropsy examination.

   F_1-generation animals that were born alive were identified by tattoo, and any pups found dead were identified with a marking pen.

On PND4, litters with a minimum of seven pups (minimum 3/sex/litter) were culled to yield - as closely as possible - four males and four females. When the number of male or female pups was less than four, a partial adjustment was made (e.g., three females and five males). When there were more than 23 acceptable litters for any dose level, surplus litters were sacrificed on PND 4 after weighing without routine necropsy, with preference given to retaining litters with a full complement of four males and four females.

F_1 animals were then randomised by a computer software program to one of four Sets designated A to D. One male and/or female per litter (approx. 16-20/sex/dietary level, at a minimum of 10/sex/dietary level, representing at least 20 litters per level) were assigned to: Motor activity (Set A); Auditory startle (Set B); Passive avoidance, water maze, and detailed observational battery (Set C). On PND 21, the whole brain was collected from a separate group of randomly selected offspring (Set D; 10/sex/dietary level; representing 20 litters per level) for micropathologic examination and morphometric analysis. Additional Set D animals (10/sex/dietary level) were retained for identical analysis at PND75, with the balance of animals assigned reserved for use as replacement animals or sacrificed without further examination (see Table KIIA 5.7.5/02 for further details of group assignment and parameter analyses).

For detailed observational battery and motor activity testing, the same individual animals were evaluated at all scheduled time points. For the selection of animals and testing paradigms for cognitive (learning and memory) assessment, the same animals were used for assessments at the weanling and adult ages, but different tests were administered at the two ages.

The dosing regimen was stated to be selected on the basis of the 2-generation reproduction study in rats (Milius, 2011) with the test substance, where at 1800 ppm substantial decreases in bodyweight gain were seen throughout the study in parent females, and decreased birthweight and decreased bodyweight gain were seen in F1 pups. At 500 ppm, effects were confined to a 20% decrease in bodyweight gain of parent females.

Table KIIA 5.7.5/01: Maternal dietary intake of test material

Conc. in diet (ppm)	Mean maternal intakes[a] (mg/kg bw/d)						Animal IDs (dams)
	Mean (GD 6 to LD 21)	Gestation [N=27 to 30]		Lactation [N=23]			
		6 to 13	13 to 20	0 to 7	7 to 14	14 to 21	
0	**0**	0					UW0101 to 0130
120	**10.3**	9.9±0.20	9.4±0.14	8.5±0.26	11.2±0.18	12.4±0.20	UW1101 to 1130
500	**42.4**	41.9±1.36	40.0±0.56	34.4±1.31	47.0±0.68	48.7±1.17	UW2101 to 2130
1200	**102**	93.5±3.29	92.7±2.42	86.3±2.18	115.7±2.00	120.1±2.71	UW3101 to 3130

Note: dietary concentrations were reduced during Weeks 1–3 of lactation by 50%, based on estimated increases in feed consumption. [a] Unadjusted for purity or potential degradation. Data excerpted from pages 12 and 41 of the study report.

Table KIIA 5.7.5/02: Detailed study Design

Parameters and groups	Dietary concentration (ppm) [*reduced by 50% during lactation wks 1 to 3*]			
	0	**120**	**500**	**1200**
Maternal animals				
No. of Maternal Animals	30	30	30	30
Detailed Observational Battery (GD 13, 20 / LD 11, 21)	30/10	30/10	30/10	30/10
Offspring				
Set C; Detailed Observational Battery [PND 4, 11, 21, 35(±1), 45(±1), 60(±2)]	20 (min. 10)/sex	20 (min. 10)/sex	20 (min. 10)/sex	20 (min. 10)/sex
Set A; Motor Activity [PND 13, 17, 21, 60(±2)]	20 (min. 10)/sex	20 (min. 10)/sex	20 (min. 10)/sex	20 (min. 10)/sex
Set B; Auditory Startle Habituation [PND 23, 60(±2)]	20 (min. 10)/sex	20 (min. 10)/sex	20 (min. 10)/sex	20 (min. 10)/sex
Set C; Learning & Memory [PND 23/30, 60 (±2)/67(±2)]	16 (min. 10)/sex	16 (min. 10)/sex	16 (min. 10)/sex	16 (min. 10)/sex
Set D; Brain Weight PND 21 PND 75(±5)	 10/sex 10/sex	 10/sex 10/sex	 10/sex 10/sex	 10/sex 10/sex
Set D; Neuropathology PND 21 PND 75(±5)	 10/sex 10/sex	 10/sex 10/sex	 10/sex 10/sex	 10/sex 10/sex

'min.' – denotes 'minimum'. Data excerpted from page 21 of the study report.

3. Dosing suspensions, preparation, and analysis

Batches of test diet were prepared weekly by solvating appropriate portions of test substance in acetone vehicle (concentration not reported) and mixing the solutions into feed to reach nominal test substance concentrations. The acetone was allowed to evaporate prior to administration. The control diet was prepared in the same way, excluding the test substance. Test diet formulations were prepared weekly, and prepared feed was stored at -22±4ºC when not in use. Achieved test substance concentrations were analysed using HPLC-UV. Analysis of stability and homogeneity of the test substance in admixed diet was not undertaken, with the study authors instead citing the stability data for 20 and 25000 ppm test substance concentrations in diet reported by Moore (2010; Report No. TXRVP027A).

During gestation, the concentration of test substance in the 120, 500 and 1200 ppm dietary levels averaged 98-102% of nominal concentrations. During lactation, the concentration of test substance in the 60, 250 and 600 ppm dietary levels averaged 98% of nominal concentrations.

The concentration of test substance in samples of diet collected from the top, middle and bottoms layers of 20 and 2500 ppm diets were 92-97% and 94-100% of nominal concentration, respectively. The concentration of test substance in samples of 20 and 2500 ppm diets were 99-105% of nominal after storage for 7 days at room temperature, and were 96-105% of or for 28 days in the freezer were 96-101% of nominal after 28 days of storage in the freezer.

4. Statistics

Statistical methods employed in this study are listed in Table KIIA 5.7.5/03, and were performed using INSTEM Computer Systems, TASC, or SAS statistical software. Statistical

significance was defined as $p \leq 0.05$, except in the case of Bartlett's Test where it was defined as $p \leq 0.001$.

Table KIIA 5.7.5/03: Statistics used in data analyses.

Parameter	Preliminary test	Method of statistical analysis	
		If preliminary test is not significant (p>0.05)	**If preliminary test is significant (p≤0.05)**
Continuous data, unless specified below	**Bartlett Test**	**One-way analysis of variance** followed by 2-sided **Dunnett's test**	**Kruskal-Wallis test** followed by **Mann-Whitney U Test.**
Detailed FOB	One-way analysis of variance **(ANOVA)**	**Dunnett's test**	
Categorical data, unless specified elsewhere	**General Linear Modeling and Categorical Modeling (CATMOD) Procedures**	**Dunnett's test; Analysis of Contrasts**	
Motor and locomotor activity	**ANOVA**	**ANOVA (session activity data); Repeated measures ANOVA (interval data)**	
Auditory startle response amplitude data (peak amplitude)	**ANOVA**	**Dunnett's test (if significant)**	
Auditory startle response amplitude data (test blocks)	**Repeated-measures ANOVA**	**Dunnett's test (if significant)**	
Passive avoidance – latency	**Wilcoxon Test**		
Passive avoidance - trials-to-criterion, **acquisition phase**	**Kruskal-Wallis and Wilcoxon Tests**		
Passive avoidance – trials-to-criterion, **retention phase**	**Fisher's Exact Test**		
Water maze - latency	Univariate **one-way analysis of variance** followed by **Dunnett's test**		
Water maze - trials-to-criterion, number of errors, **acquisition phase**	**Kruskal-Wallis** and **Wilcoxon Tests**		
Water maze - trials-to-criterion, number of errors, **retention phase**	**Fisher's Exact Test**		
Organ weights Gross brain morphology	**Bartlett Test**	**One-way analysis of variance (if data were homogenous)**	**Kruskal-Wallis test (if data were not homogenous)**
Microscopic brain morphology	**2-tailed t-Test** for two-group comparisons; **ANOVA followed by Dunnett's Test** for multiple-group comparisons		
Ophthalmology Gross Pathology	**Chi-Square** and **one-tailed Fisher's Exact Tests (only if visual examination of data suggested potential treatment-related effects)**		
Histopathology	**Chi-Square Fisher's Exact Test**		

C. METHOD

1. Observations including Detailed Observational Battery and Neurobehavioral Evaluations

General (parental animals and offspring)

In general, following acclimation parental animals were observed at least once daily for clinical signs of toxicity until removal from the study (females) or completion of co-housing (males). Observers had the discretion to remove animals from cages and subject them to detailed physical examinations.

As soon as possible after parturition, pups were examined for ano-genital distance to establish their sex. Live pups were counted, sexed and weighed individually for each litter on PND 0, 4, 11, 17 and 21.

Daily (a.m.) throughout lactation, offspring were examined cageside for gross signs of mortality or moribundity. After weaning on LD 21, offspring were observed for survival on a daily basis and observed for clinical signs on a weekly basis. Any gross signs of toxicity in the offspring were recorded as they were observed, including the time of onset, degree, and duration.

Routine daily observations for clinical signs of toxicity and physical examinations were undertaken on all maternal animals from GD 6 and offspring (LD 1 to LD 21), under conditions of blinding to dosing group levels.

Detailed Observational Battery

Detailed Observational Battery examinations were undertaken for dams on GD 13 and 20, and for both pups and remaining dams (minimum of 10 dams/dose) on LD 11 and 21.

Detailed Observational Battery examinations comprised of the following:

- ***Signs of autonomic function, including:***
  1) Ranking of degree of lacrimation and salivation, with range of severity scores from none to severe
  2) Presence or absence of piloerection and exophthalmos
  3) Ranking or count of urination and defecation, including polyuria and diarrhoea
  4) Pupillary function such as constriction of the pupil in response to light, or a measure of pupil size
  5) Degree of palpebral closure, e.g., ptosis
- ***Description, incidence, and severity of any convulsions, tremors, or abnormal movements***
- ***Description and incidence of posture and gait abnormalities***
- ***Description and incidence of any unusual or abnormal behaviours***, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may have facilitated interpretation of the data

Offspring only examinations

Beginning on PND 4, selected males and females were examined daily for surface righting, with the age of onset recorded. On PND 21, all pups were tested for the presence of pupil

constriction. From PND 29 onwards, female offspring were examined daily for vaginal patency and from PND 38, male offspring were examined daily for balanopreputial separation.

Additionally, offspring were subjected to Neurobehavioral Evaluations over PND 4 to 67 as summarised below.

Detailed Observational Battery (Set C), PND 4 to 60:

On PND 4, 11, 21, 35 (±1 day), 45 (±1 day), and 60 (±2 days), approx. 20 offspring/sex/group (representing at least 20 litters per level) were examined outside the home cage using procedures described above. PND4 and PND11 animals were examined in the open field only by necessity based on observer discretion.

Motor Activity Testing (Set A), PND 13 to 60:

Motor activity was measured for approx. 20 rats/sex/dose (representing at least 20 litters per level) on PND 13, 17, 21, and 60 (±2 days). These animals were evaluated in the figure-eight maze for 60 min at each time point, using a computer-automated system (Universal Maze Monitoring System, Version 1.41, Columbus Instruments, USA) and automated data collection. Each maze consisted of a series of inter-connected alleys (approximately 10 x 10 cm in cross-section) converging on a central arena and covered by transparent acrylic plastic. Each maze has eight infrared emitter/detector pairs to measure activity and an activity count was registered for each instance of beam interruption. Motor and locomotor activities were examined as total activity counts (beam interruptions) for the 60 min session and as activity during each 10 min interval. Motor activity was measured as the number of beam interruptions that occur during the test session. Locomotor activity was measured by eliminating consecutive counts for a given beam. Habituation was evaluated as a decrement in activity over consecutive intervals of the test session. Absorbent paper was used as flooring and replaced at the end of each day. Broad-spectrum background noise [74 ± 2dB(A)] was provided throughout the test to minimize acoustical variations during testing.

Auditory Startle Reflex Habituation (Set B), PND 23 to 60:

Auditory startle reflex habituation testing was performed in approx. 20 rats/sex/dose (representing at least 20 litters per level) on PND 23 and 60 (±2 days). A personal computer was used to control the operation of an integrated startle response test system (Coulbourn Acoustic Startle, Version 3.210-00, Coulbourn Instruments, Allentown, PA) and for automated data collection. Groups of four animals (maximum) were tested simultaneously within each of two startle system enclosures. Each enclosure was ventilated, lined with sound-attenuating and vibration-absorbing material, and housed a speaker mounted in a central position within the ceiling of the enclosure to provide the eliciting stimulus (‘S2’) - a 50 msec burst (0 msec rise/fall) of broad-spectrum "white" noise of approx. 118 dB(lin). Each enclosure also housed four load cell/force transducer assemblies that measured the startle response. During the test session, animals were placed into individual restraining cages that are positioned on top of each load cell. The test session consisted of 50 trials that began following a ~5 min adaptation period at ambient noise levels. The rats were then presented with the startle-eliciting stimulus at 10 second intervals. The average response amplitude and the magnitude of decrease (habituation) over blocks of ten trials were compared among the dosage groups. Data collection began with the presentation of S2 and continued thereafter for 200 msec. The analog signal for each response output (measured in mV) was digitized at one kHz and converted to grams

using a previously determined calibration curve for each load cell. Peak response amplitude (g) and latency (msec) measurements were taken from each animal's individual response curve. Baseline was defined as the average force (g) exerted on the platform during the first 8 msec following the onset of S2, a time period that precedes response onset. This baseline value was taken to represent an approximate body weight measurement that was used to verify that the equipment used to measure the response amplitude was functioning properly. Response amplitude was defined as the maximum value of the average curve, minus the baseline (i.e., removing the animal's body weight from the measurement). Latency to peak was defined as the time (msec) following the onset of S2 when the peak response amplitude occurs.

Learning and Memory Testing (Set C), PND 23/30 and 60/67:

Learning and memory testing were performed in approx. 16 rats/sex/dietary level, representing at least 20 litters per level. The same set of animals were used for testing passive avoidance (on PND 23 and 30) and water maze [PND 60 (± 2 days) and 67].

Passive Avoidance, PND 23 and 30: Animals were tested for acquisition on PND 23 and for retention on PND 30. Testing was conducted using equipment and computer programs from Coulbourn Instruments (Graphic State Notation 2 Version 2.002-00, USA). Testing took place in individual isolation cubicles, each housing a single shuttle cage.
The shuttle cage consisted of a Plexiglas and stainless-steel rectangular chamber fitted with front-loading access. Each shuttle cage (15 inches wide x 7.25 inches deep) was separated into two compartments of equal size (approx. 7 x 7 inches) by a wall that supports a centrally-located sliding (guillotine-type) door. The two compartments were identical, except that the walls in one compartment were lined with black film (dark-side) and the walls in the other compartment were not lined and it was illuminated during the test with a high intensity lamp. The lamp was switched on to illuminate the light compartment at the start of each trial and remained on until either the animal crossed to the dark compartment or the trial ended. The floor of the cage consisted of a grid of stainless-steel bars. The movement of the animal from the starting (light) side to the dark compartment was detected by a photocell system. A Coulbourn solid-state scanning shock generator was used to deliver a brief (0.5 second) pulse of mild (0.5 mA) distributed shock to the grid floor when the animal crossed to the dark compartment.

After adaptation, individual animals were placed individually into the “lighted” compartment of the shuttle cage, facing toward the light. After approx. 60 seconds, the trial began with the light being illuminated to signal the beginning of the trial and the door separating the two compartments opening, so that each rat was provided access to the non-illuminated side of the cage. When the rat crossed into the dark compartment, the door automatically closed, the shock was delivered, and the light switched off - signalling the end of that trial. At that time, the animal was returned promptly to the holding cage to wait for the next trial. If the rat failed to cross within 180 seconds, it was returned to the holding cage and the latency assigned an arbitrary score of 180. The procedure was repeated until either the rat remained in the lighted compartment for 180 seconds on two consecutive trials or until 15 trials elapsed. Rats that failed to meet the criterion during the learning phase were assigned a value of 15 for the trials-to-criterion variable. The test was repeated seven days later, whereupon rats were placed in the illuminated side of the apparatus, given a 60 second acclimation period, and the latency to enter the dark side was recorded. Animals that either failed to reach criterion performance within 15 trials or failed to cross to the dark compartment during the first two trials during acquisition were excluded from

the retention phase of the experiment. The dependent measures were the number of trials-to-criterion, latency to cross on Trial 1 and Trial 2 (learning phase only), and the number of rats/group that failed to reach criterion within 15 trials (learning phase only).

Water Maze, PND 60 and 67: Only animals that demonstrated acquisition on PND 60 were tested for retention (PND 67). The M-mazes were constructed of opaque Plexiglass, with corridors approx. five inches wide and walls approx. 16 inches high, with approx. 7.5 inches of water at 22 ± 1 °C. On each test trial, the rat was placed into the starting position at the base of the M-maze stem, located between the two lateral arms. On the first (learning) trial, the rat was required to enter both arms of the maze before being provided access to the exit ramp to escape the water and then removed from the maze. The initial arm chosen on this learning trial was designated the incorrect goal during the subsequent 15 trials (maximum). Rats that failed to make a correct goal choice within 60 seconds in any given trial were guided to the correct goal with the exit ramp and then removed from the water. Between trials, the animal was returned to a transport cage to wait for the next trial. The inter-trial interval was approx. 15 (±5) seconds. Each rat was required to reach a criterion of five consecutive errorless trials to terminate the test session. The maximum number of trials in any test session was fifteen. Latency (in seconds) to choose the correct goal or the maximum 60 second interval was recorded for each trial, as well as the number of errors (incorrect turns in the maze) during each trial. Animals that satisfied the above criteria within the 15 trial limit were tested for retention seven days following acquisition. Dosage groups were compared for the following dependent measures: measures for acquisition include the number of trials-to-criterion, the average number of errors (incorrect turns in the maze) for each trial and the latency to reach the correct goal on trial 2 (a measure of short-term retention). Measures for retention include the number of trials-to-criterion, the average number of errors for each trial, and the latency (in seconds) to reach the correct goal on trial 1 (a measure of long-term retention).

2. Bodyweights

All dams were weighed once immediately after acclimation, once on GD 0, 6, 13 and 20 and LD 0, 4, 7, 14 and 21.

Live pups were weighed individually for each litter on PND 0, 4, 11, 17 and 21, then weekly thereafter. Animals were also weighed when vaginal patency or balanopreputial separation was first evident.

3. Food consumption

Food consumption was measured over the following intervals: GD 6-13, GD 13-20, LD 0-7, LD 7-14 and LD 14-21. The study director noted that measures of food consumption may have included consumption by the pups, particularly during the third week of lactation. Food consumption was not measured for pups.

4. Ophthalmological examinations

At between PND 50–60, a minimum of 10 rats/sex/dietary level, representing at least 20 litters per level, selected from Sets A, B, and C were subjected to ophthalmological examination of each eye proceeded by examining the pupillary reflex using a penlight or transilluminator. Thereafter, mydriatic agent was instilled into each eye and an indirect ophthalmoscope equipped with a condensing lens was used to examine vitreous humor, retina, choroid, and optic disc and the lens. Additionally, the conjunctiva and cornea were examined either before

or after pupillary dilatation. These animals selected for ophthalmological examination were also selected for perfusion at study termination for histopathological examination.

5. Sacrifice and pathology

Culled dams and pups were sacrificed by CO_2 asphyxiation and decapitation, respectively, without necropsy.

Following the weaning of their respective litters, dams with litter were sacrificed on LD 21, by CO_2 asphyxiation. Females that were sperm positive and/or had an internal vaginal plug but did not deliver were sacrificed on or after GD 24 without necropsy.

Necropsy groups

10 pups/sex/dose from sets A, B, and C, representing animals from at least 20 litters/dose, were sacrificed by CO_2 asphyxiation on PND 70 to 80 and subjected to necropsy that included measurement of fresh brain weights and examining all organs, body cavities, cut surfaces, external orifices and surfaces, and recording gross abnormalities. Remaining animals in sets A to C were discarded without necropsy or further examination of tissues. Animals selected for brain weight/neuropathological examination from Set D were sacrificed on PND 21 or PND 70–80 (see below).

Perfusion groups

Offspring selected for *in situ* perfusion were weighed then deeply anesthetized by a ~50 mg/kg bw i.p. dose of pentobarbital and perfused via the left ventricle with a sodium nitrite (in phosphate buffer) flush followed by universal fixative [4% (w/v) EM grade formalin] in phosphate buffer. On PND 21 (Set D), only the brain was collected and post-fixed. For Set A–C offspring selected for perfusion at study termination, the brain together with spinal cord, both eyes, selected peripheral nerves (sciatic, tibial, and sural), gasserian ganglion, gastrocnemius muscle, and both forelimbs, was post-fixed in 10% buffered formalin. An exception was the eyes, which were post-fixed in Davidson's fixative.

Prior to perfusing the brain, an individual with knowledge of each animal's dosing regimens used a vernier caliper to undertake morphometry analysis to measure the following:

- the Anterior-to-posterior length of the cerebrum, extending from the anterior pole to the posterior pole, exclusive of the olfactory bulbs; and,

- the Anterior-to-posterior length of the cerebellum, extending from the anterior edge of the cortex to the posterior pole.

Perfused brains were then sliced into eight coronal sections, embedded in paraffin, sectioned into approx. 5 µm, and stained using hematoxylin and eosin (H&E) for histological examination. For morphometry analysis, duplicate brain section samples were stained with luxol fast blue/cresyl violet.

Morphometry analysis of sections was performed by the same individual as the gross measurements (i.e. the anterior-to-posterior length of the cerebrum and cerebellum) and consisted of the following three linear measurements:

- Forebrain (Bregma -0.12 and -0.36 mm) frontal cortex thickness, as dorsal portion of the cerebral cortex within the coronal section passing through the region of the optic chiasm;

- Midbrain (Bregma -3.24 and -3.48 mm) hippocampal gyrus thickness, as full width of the hippocampal gyrus, from the ventral tail of the dentate gyrus to the overlying subcortical white matter;

- Cerebellum height (Bregma -11.76 and -12.00 mm), as the roof of the fourth ventricle to the dorsal surface.

Morphometry analysis was performed using the same structures and areas for both Day 21 and Day 70 rats and undertaken initially in high dose and control animals, and in mid dose groups only if suspected test substance abnormalities were observed.

Acceptability and rejection criteria for morphometry slide usage were based on definitions of Bregma numbers detailed in *"The Rat Brain in Stereotaxic Coordinates", Paxinos and Watson (6th edition),* along with the quality of sectioned tissues (i.e. damaged or not of an adequate transverse nature). Re-sectioning of tissue was undertaken as necessary to fulfil acceptance criteria.

Additionally, histopathological analysis was conducted on all brain sections, three levels of the spinal cord (cervical, thoracic, and lumbar), the cauda equina, eyes, optic nerves, and gastrocnemius muscle, by embedding these in paraffin and staining with H&E. Cervical and lumbar swelling dorsal root ganglia (including dorsal and ventral root fibers) and gasserian ganglia were embedded in glycol methacrylate (GMA), sectioned at 2-3 µm, and stained using a modified Lee's stain. Sciatic, tibial, and sural nerves were embedded in GMA resin and sectioned longitudinally, and the sciatic nerve was also cut in cross section. Analysis proceeded first in selected control and high dose animals, and was not undertaken in mid-dose animals in the absence of abnormal findings.

6. Measurement indices and outcomes

The following reproductive parameters were reported:

Mating Index = (no. of inseminated females ÷ no. of females co-housed with males) × 100

Fertility Index = (no. of pregnant females ÷ no. of inseminated females) × 100

The following offspring viability parameters were reported:

Live Birth Index = (no. of live pups born per litter ÷ total no. of pups per litter) × 100

Viability Index = (no. of live pups on Day 4 pre-culling per litter ÷ no. of live pups born per litter) × 100

Lactation Index = (no. of live pups on Day 21 per litter ÷ no. of live pups on day 4 post-culling per litter) × 100

II. RESULTS AND DISCUSSION

A. MATERNAL EFFECTS

1. Clinical signs of toxicity and mortality

No treatment-related clinical signs of toxicity were observed in any maternal group, including over the periods of gestation and lactation. Minor clinical signs (Table KIIA 5.7.5/03) were considered incidental.

There were no instances of premature mortality in maternal animals.

Table KIIA 5.7.5/03: Clinical signs and mortality in maternal animals

Parameter	Dose (ppm)			
	0	**120**	**500**	**1200**
GD 6 to 24				
No examined on GD 6	**30**	**30**	**30**	**30**
Red vaginal discharge	0	0	1	0
Hair loss	1	1	2	3
Fused digits	1	0	0	0
No. found dead	0	0	0	0
LD 0 to 21				
No examined on LD 0	**27**	**29**	**29**	**30**
Hair loss	1	1	3	4
Fused digits	1	0	0	0
No. found dead	0	0	0	0

The numbers of dams with no pups by GD 24 were 3, 1, 1, and 0 for the controls, low-, mid- and high-dose groups, respectively. Data excerpted from page 38 of the study report.

2. Detailed Observational Battery - dams

Abnormal findings for dams in the Detailed Observational Battery were confined to statistically significant increases in the mean number of urination pools in the open field for mid- and high-dose females on LD 11 (3.7 and 2.6, respectively, relative to the controls (0.6 for controls). The study authors regarded this finding not to be test substance-related, due to the absence of dose dependency and presence at only one time point. The evaluator agreed with the study author's conclusion. There was also a statistically significant increase in the number of dams in the 1200 ppm dose group with alopecia when compared to controls on LD 11 and 21 (3 versus 0 in all other dose groups); this finding was not considered to be toxicologically significant as alopecia was observed in 0 to 3 animals from various dose groups throughout gestation.

3. Body weight, body weight gain, and food consumption

During gestation, statistically significant differences in bodyweights were confined to 1200 ppm (Table KIIA 5.7.5/04), where a 21% decrease in mean bodyweight gain over the gestation period (GD 0–20) and a 7% lower mean final bodyweight relative to controls was seen at GD 20 (both $p \leq 0.01$).

During lactation, statistically significant ($p \leq 0.05$) changes in bodyweights were again confined to 1200 ppm (Table KIIA 5.7.5/05), where a 4% lower bodyweight relative to controls was seen at LD 0 and a 6% decrease relative to controls was seen at LD 4. These differences remained as 4 to 6% lower terminal weights relative to control up to LD 21, but had lost statistical significance from LD7 onwards (*i.e.*, p was >0.05). Overall bodyweight gain over the lactation period was reduced by 34% in the 500 ppm and 23% in 1200 ppm groups, but a dose-response was not

observed, and no significant effect on bodyweight at the end of lactation was apparent in the 500 ppm dose group, which demonstrated the largest reduction in bodyweight gain when compared to controls. The overall bodyweight gain from LD 0 to 21 was 7%, 8%, 5%, and 6% of the initial mean bodyweight values on LD 0 for the 0, 120, 500, and 1200 ppm dose groups, respectively.

The study author regarded food consumption (as g/animal/day) to be unaffected over both gestation and lactation. The primary reviewer r noted a marginal decrease of 5 to 8% in food consumption over GD 6 to 13 across treated groups, and a 5% decrease from GD 13 to 20 seen in the 1200 ppm group relative to controls. While the GD 6–13 observation appears to be treatment-related, the lack of a statistically significant effect on body weight at 120 and 500 ppm over the gestation period suggests that the effect is not toxicologically adverse at these dose levels, but should be considered adverse at 1200 ppm noting the 21% decrease in body weight gain. The secondary reviewer agreed with the study author that food consumption was unaffected by treatment during the lactation period.

Overall, the US EPA secondary reviewer concluded that the slight body weight changes seen in the dams of all dose levels did not approached the magnitude which could be considered as adverse.

Table KIIA 5.7.5/04: Bodyweight and food consumption during gestation

	Dose (ppm)			
	0	**120**	**500**	**1200**
Mean bodyweight (g)				
GD 0	206.4±2.18	208.0±2.54 (+0.8%)	209.6±2.36 (+1.6%)	209.8±2.59 (+1.6%)
GD 6	229.1±2.71	230.8±2.96 (+0.7%)	231.2±3.46 (+0.9%)	233.1±2.72 (+1.7%)
GD 13	254.5±3.74	256.0±3.58 (+0.6%)	256.0±3.27 (+0.6%)	245.3±2.92 (-3.6%)
GD 20	320.9±4.09	314.7±5.14 (-1.9%)	318.7±4.26 (-0.7%)	299.9±4.05** (-6.5%)
$\bar{x}$ bodyweight gain GD 0-20	114.5±2.80	107.5±3.38 (-6.1%)	109.1±2.59 (-4.7%)	90.3±2.57** (-21.1%)
Mean food consumption (g/animal/day)				
GD 6 to 13	20.1±0.73	19.1±0.47 (-5.0%)	18.9±0.47 (-6.0%)	18.4±0.59 (-8.5%)
GD 13 to 20	20.3±0.37	20.1±0.43 (-1.0%)	20.2±0.40 (-0.5%)	19.2±0.48 (-5.4%)

** Statistically significant at $p \leq 0.01$. Data excerpted from page 40 of the study report, except for per cent change from control, which was calculated by the evaluator using Microsoft Excel.

Table KIIA 5.7.5/05: Maternal bodyweight and food consumption during lactation

	Dose (ppm)			
	0	**120**	**500**	**1200**
Mean bodyweight (g)				
LD 0	244.0±3.30	245.2±3.85 (+0.5%)	242.9±3.43 (-0.5%)	233.3±2.76* (-4.4%)
LD 4	262.0±3.19	256.7±4.48 (-2.0%)	256.1±3.16 (-2.3%)	247.2±3.62** (-5.6%)
LD 7	265.4±2.98	263.2±3.89 (-0.8%)	263.4±3.36 (-0.8%)	255.7±3.46 (-3.7%)
LD 14	279.6±3.17	274.6±4.05	275.2±3.30	266.6±2.86

		(-1.8%)	(-1.6%)	(-4.6%)
LD 21	261.1±5.87	264.1±4.50 (+1.1%)	254.2±3.88 (-2.6%)	246.5±6.38 (-5.6%)
Bodyweight gain LD 0-21	17.1	18.9	11.3 (-34%)	13.2 (-23%)
Mean food consumption (g/animal/day)				
LD 0 to 7	36.0±1.05	35.3±1.01 (-1.9%)	34.1±1.14 (-5.3%)	34.0±0.80 (-5.6%)
LD 7 to 14	50.8±0.84	50.3±1.08 (-1.0%)	50.7±0.86 (-0.2%)	50.3±1.00 (-1.0%)
LD 14 to 21	55.9±1.76	58.1±1.12 (+3.9%)	54.7±1.06 (-2.1%)	54.5±1.35 (-2.5%)

* Statistically significant at * $p \leq 0.05$ or ** $p \leq 0.01$ level. Data excerpted from page 40 of the study report, except for per cent change from control, which was calculated by the evaluator using Microsoft Excel, and bodyweight gain which was calculated by the evaluator from the mean bodyweight values on LD 21 and LD 0.

4. Reproductive outcomes

There were no adverse test substance-related effects on reproductive parameters or litter data endpoints during the course of this study (Table KIIA 5.7.5/06).

Table KIIA 5.7.5/06: Reproductive performance

Parameter	Dose (ppm) [N=30]			
	0	**120**	**500**	**1200**
No. not pregnant	3	1	1	0
No. of dams that delivered dead pups	1 (1 pup)	0	0	2 (1 pup/litter)
No. of dams with pre-mature delivery	0	0	0	0
Mating index	100	100	100	100
Fertility index (No. of pregnant females/No. of inseminated females X 100)	**90.0**	**96.7**	**96.7**	**100**
Mean gestation length (days)[a] **Range[a]**	21.7±0.10 (21.0-22.0)	21.7±0.13 (20.0-23.0)	21.8±0.13 (21.0-23.0)	21.9±0.16 (20.0-23.0)
Median gestation length (days)[a]	[22.0]	[22.0]	[22.0]	[22.0]

No findings were statistically significant at the $p \leq 0.05$ level. Data excerpted from page 42 of the study report.
[a] The mean values for gestation length are based on the results from the 23 dams per dose level retained on study (i.e., values for litters sacrificed on PND 0 or PND 4 are not included).

B. OFFSPRING EFFECTS

1. General viability

No test substance-related effect on litter size or pup viability was observed at any dose level (Table KIIA 5.7.5/07). Of the litters that were sacrificed on PND 4 and not included in Table K11A 5.7.5/07, only one litter each from the 500 and 1200 ppm dose groups had dead or missing pups. The litter from the 500 ppm dose group had 1 pup missing on PND 1 and 2 pups missing on PND 2, while the litter from the 1200 ppm dose group had 1 dead pup on PND 1. The exclusion of these results from the tabulated data in the table below does not impact on the conclusions pertaining to viability.

Table KIIA 5.7.5/07: Summary of Offspring Effects

Parameter	Dose (ppm)			
	0	**120**	**500**	**1200**
No. of litters	23	23	23	23
Total No. of pups born	269	265	275	255
Total No. of pups missing	0	0	0	1 (PND 5)

Parameter	Dose (ppm)			
	0	**120**	**500**	**1200**
Litters with pups missing	0	0	0	1
Total No. of pups found dead	2 (PND 0 & 4)	2 (PND 1 & 2)	0	0
Litters with pups found dead	2	2	0	0
Total No. of pups cannibalised	0	0	0	0
Litter with pups cannibalised	0	0	0	0
Litter size [median] (range)	**11.7±0.34** [12.0] (8.0-15.0)	**11.5±0.39** [12.0] (8.0-14.0)	**12.0±0.36** [12.0] (8.0-15.0)	**11.1±0.38** [11.0] (7.0-14.0)
Stillborn pups **Number** % Mean±S.E. [Median] (Range)	 **1** 0.4 0.0±0.04 [0.0] (0.0-1.0)	 **0** 0.0 0.0±0.00 [0.0] (0.0-0.0)	 **0** 0.0 0.0±0.00 [0.0] (0.0-0.0)	 **2** 0.8 0.1±0.06 [0.0] (0.0-1.0)
Mean No. of viable pups **Birth** Day 4 (Pre-cull) Day 4 (Post-cull) Day 21	 **12** 12 8 8	 **12** 11 8 8	 **12** 12 8 8	 **11** 11 8 8
Live Birth Index [mean ± S.E] [median] (range)	**99.6±0.43** [100.0] (90-100)	**100.0±0.0** [100.0] (100-100)	**100.0±0.0** [100.0] (100-100)	**99.3±0.51** [100.0] (91-100)
Viability Index [mean ± S.E.] [median] (range)	99.3±0.51 [100.0] (91-100)	99.1±0.64 [100.0] (89-100)	100.0±0.0 [100.0] (100-100)	100.0±0.0 [100.0] (100-100)
Lactation Index [mean ± S.E.] [median] (range)	100.0±0.0 [100.0] (100-100)	100.0±0.0 [100.0] (100-100)	100.0±0.0 [100.0] (100-100)	99.5±0.54 [100.0] (88-100)

There were no statistically significant differences from control at the $p < 0.05$ level.
Data excerpted from page 43 of the study report.

2. Clinical signs of toxicity and mortality in offspring

   No treatment-related clinical signs of toxicity were observed in any group, including over the pre-weaning and post-weaning periods.

   Incidental findings without dose dependency noted in various dose groups included hair loss and bruising noted during the pre-weaning period, and yellow perigenital stain, red lacrimal stain, red eye, exophthalmia, skin missing on tip of tail, and minor scabs or scars noted during the post-weaning period.

   Following the standardization on PND 4, mortality was confined to one female pup at 1200 ppm that was missing on PND 5. As it occurred in only one litter, and is a relatively common finding, the study authors regarded it to be unrelated to the test substance. The evaluator agrees with the study author's conclusion.

3. Detailed Observational Battery - pups

There were no treatment-related findings in the cage-side and open field assessments or observations during handling conducted between PND 4 and PND 60.

Pups - Session motor and locomotor activity

A non-statistically significant (*i.e.*, p was >0.05) increase in both motor (114±130 counts) and locomotor activity (20±39) was seen in males at 1200 ppm on PND 13 relative to concurrent controls (55±55 and 5±8, respectively). The study authors regarded these to be test substance-related, but also partially due to a lower mean average activity for concurrent controls versus PND 13 historical controls (59±58 to 72±40 for motor activities and 6±8 to 11±10 for locomotor activities in the last five studies conducted at the laboratory) (Table KIIA 5.7.5/08 and KIIA 5.7.5/09). Aside from high-dose males on PND 13, all test groups exhibited appropriate habituation to the motor activity testing environment over the test interval (data not shown). Males from the 1200 ppm dose group exhibited a sustained higher level of motor and locomotor activity over the testing interval.

All other motor and locomotor activity findings were regarded by the study authors to be unrelated to the test substance due to poor dose dependency, poor time concordance, and/or minor extent of change.

Table KIIA 5.7.5/08: Motor Activity Data (total activity counts for session)

PND	Dose (ppm) [N=20[A]]								$\bar{x}$ over 5 previous studies	
	0	120	500	1200	0	120	500	1200	HC	
	M				F				M	F
Total activity counts for session [Mean (±S.D.)]										
PND 13	55±55	66±82 (+20%)	63±66 (+15%)	114±130 (+107%)	74±141	88±81 (+19%)	68±60 (-8%)	62±40 (-16%)	**68±5**	**NA**
PND 17	146±117	260±104 (+78%)	173±114 (+18%)	221±144 (+51%)	201±152	149±112 (-26%)	243±121 (+21%)	227±200 (+13%)	**194±61**	
PND 21	307±119	367±132 (+20%)	331±149 (+8%)	367±123 (+20%)	303±88	333±140 (+10%)	308±132 (+2%)	322±119 (+6%)	**302±30**	
PND 60	503±142	518±108 (+3%)	505±121 (+0%)	529±111 (+5%)	656±143	666±233 (+2%)	624±202 (-5%)	790±232 (+20%)	**552±37**	**697+28**

No changes were statistically significant at the p≤0.05 level. 'HC' – historical control average over five previous studies in the laboratory and provided as summary results only and not subjected to statistical analysis of difference to results of the current study. 'NA' – denotes not available, as the only motor activity timepoint provided for HC females was PND 60. 'A' – except for females at 120 ppm, where N=19. Data excerpted from page 50 of the study report, except for per cent change from control, which was calculated by the evaluator using Microsoft Excel.

Table KIIA 5.7.5/09: Locomotor Activity Data (total activity counts for session)

PND	Dose (ppm) [N=20[A]]								$\bar{x}$ over 5 previous studies	
	0	120	500	1200	0	120	500	1200	HC	
	M				F				M	F
Total activity counts for session [Mean (±S.D.)]										
PND 13	5±8	9±11 (+80%)	7±17 (+40%)	20±39 (+300%)	6±11	8±12 (+33%)	12±21 (+100%)	7±5 (+17%)	8±2	**NA**
PND 17	34±27	66±33 (+94%)	47±30 (+38%)	57±42 (+68%)	53±46	37±32 (-30%)	65±40 (+23%)	53±38 (+0%)	48±19	

PND 21	87±28	113±28 (+30%)	93±39 (+7%)	99±35 (+14%)	103±34	91±40 (-12%)	93±36 (-10%)	99±37 (-4%)	88±11	
PND 60	344±124	343±93 (+0%)	340±105 (-1%)	344±89 (+0%)	425±97	433±176 (+2%)	394±147 (-7%)	533±176 (+25%)	383±30	467±26

No changes were statistically significant at the p≤0.05 level. See previous table for description of terms. 'Δ cntrl (%)' – denotes per cent change from control. 'A' – except for females at 120 ppm, where N=19. Data excerpted from page 51 of the study report, except for per cent change from control, which was calculated by the evaluator using Microsoft Excel.

Auditory Startle Habituation

On PND 60, overall startle amplitude for all 50 trials was statistically significantly (p≤0.05) increased by approx. 2-fold in females at 1200 ppm relative to controls (156 g versus 78 g). This was associated with a non-statistical increase in response amplitude in test block 1 (i.e. trials 1–10) and a statistically significant increase in test blocks 2 to 5 (i.e. trials 11–50) relative to control (Table KIIA 5.7.5/10a). This finding is considered test substance related. There were no other test substance related findings in startle amplitude. There were no test-substance related findings in time to peak amplitude (Table KIIA 5.7.5/10b). **Table KIIA 5.7.5/10a: Auditory Startle Habituation – Peak Amplitude (g)**

PND	**Block No's**	**Dose (ppm) [N=20/group]**								**HC**	
		0	**120**	**500**	**1200**	**0**	**120**	**500**	**1200**		
		M				**F**				**M**	**F**
23	**1**	44±18	48±13	43±18	46±22	39±22	38±16	35±21	45±17	NA	NA
	2	43±16	50±13	42±23	41±15	39±22	36±16	36±18	43±17		
	3	39±16	48±15	40±23	42±14	36±19	37±18	34±18	43±17		
	4	40±17	48±14	38±17	41±15	32±14	37±17	31±18	39±16		
	5	37±16	45±15	37±14	35±12	29±15	34±15	28±14	36±16		
	1 to 5 $\bar{x}$	41±15	48±12	40±18	41±13	35±17	36±14	33±17	41±15		
	BW (g)	60	60	60	57	57	58	56	57		
60	**1**	270±115	301±159	241±151	324±162	102±74	133±75	140±91	186±129		129±17
	2	255±161	299±190	203±141	289±215	94±82	121±94	127±84	**181±126***		138±19
	3	200±140	245±157	175±136	214±183	71±73	108±86	113±76	**160±116***		107±16
	4	187±119	214±140	152±130	177±163	68±54	84±58	96±65	**132±87***		86±12
	5	145±92	187±131	134±116	144±108	58±33	74±46	76±50	**122±83***		70±9
	1 to 5 $\bar{x}$	211±115	249±145	181±127	230±153	78±57	104±65	110±68	**156±102* (+100%)**		106±11
	BW (g)	292	291	280	285	185	180	184	174		**NA**

'NA' – not available, as HC data was provided for only for females on PND 60. '*' - statistically significantly (p≤0.05) different from concurrent control. 'HC' – historical control, $\bar{x}$ over 5 studies. Data excerpted from page 53 of the study report except for per cent change from control, which was calculated by the evaluator using Microsoft Excel.

Table KIIA 5.7.5/10b: Auditory Startle Habituation – Time to peak amplitude (msec)

PND	**Block No's**	**Dose (ppm) [N=20/group]**							
		0	**120**	**500**	**1200**	**0**	**120**	**500**	**1200**
		M				**F**			
23	**1**	37±3	37±4	37±4	36±3	38±5	37±6	40±6	36±3
	2	37±3	36±5	39±5	37±4	36±5	37±5	39±8	36±4
	3	36±4	36±7	38±6	36±3	36±4	37±4	38±7	35±3
	4	35±3	34±4	36±4	35±3	36±4	37±4	38±8	35±3
	5	38±4	36±4	38±7	35±3	39±7	35±3	41±6	36±4
	1 to 5 $\bar{x}$	37±2	36±4	38±4	36±2	37±3	36±3	39±6	35±3
	BW (g)	60	60	60	57	57	58	56	57
60	**1**	40±2	41±3	41±4	41±2	44±5	41±8	42±5	40±5
	2	39±2	39±3	39±3	39±4	41±4	41±5	42±6	39±3

	3	38±3	38±3	38±3	39±3	38±6	39±4	40±6	38±4
	4	38±3	38±4	40±5	39±2	39±4	39±5	41±6	39±5
	5	38±4	38±3	39±4	39±3	39±6	37±4	40±5	38±4
	1 to 5 $\bar{x}$	39±2	39±3	40±3	40±2	40±3	39±4	41±4	39±3
	BW (g)	292	291	280	285	185	180	184	174

Data excerpted from pages 194 to 199 of the study report**.**

Learning and memory testing: Postweaning, passive avoidance and water maze

Acquisition and retention parameters for the passive avoidance and water maze tasks were unaffected by dosing (Table KIIA 5.7.5/11).

Table KIIA 5.7.5/11a: Passive Avoidance Performance at PND 23 and 30 (mean ± S.D.)

Session/Parameter		**Dietary Level**			
		Control	**120 ppm**	**500 ppm**	**1200 ppm**
Males					
Session 1 (Learning Phase)	Number of animals tested	16	16	16	16
	Number of animals included in analysis	16	16	16	16
	Trials to criterion	3.1±0.3	3.3±0.9	3.9±3.0	3.1±0.3
	Latency trial 1 (seconds)	37.4±28.8	32.5±41.6	21.2±21.2	33.0±27.6
	Latency trial 2 (seconds)	172.1±31.8	163.9±44.5	154.4±55.0	174.7±21.3
	Failed to meet criterion	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Failed to cross during learning phase	0 (0%)	1 (6%)	0 (0%)	0 (0%)
Session 2 (Retention Phase)	Number of animals tested	16	16	16	16
	Number of animals included in analysis	16	15	16	16
	Trials to criterion	2.3±0.6	2.7±1.0	2.4±0.8	2.0±0.0
	Latency trial 1 (seconds)	169.0±30.5	156.6±61.9	149.6±59.9	180.0±0.0
	Latency trial 2 (seconds)	178.0±7.9	160.7±30.0	180.0±0.0	180.0±0.0
Females					
Session 1 (Learning Phase)	Number of animals tested	16	16	16	16
	Number of animals included in analysis	16	16	16	16
	Trials to criterion	3.3±0.8	3.0±0.4	3.1±0.3	3.0±0.0
	Latency trial 1 (seconds)	44.6±41.9	53.3±57.4	35.5±34.0	22.7±20.5
	Latency trial 2 (seconds)	166.0±41.0	178.4±6.4	166.1±37.9	180.0±0.0
	Failed to meet criterion	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Failed to cross during learning phase	0 (0%)	1 (6%)	0 (0%)	0 (0%)
Session 2 (Retention Phase)	Number of animals tested	16	16	16	14
	Number of animals included in analysis	16	15	16	14
	Trials to criterion	2.4±0.6	2.6±0.9	2.3±0.6	2.2±0.6
	Latency trial 1 (seconds)	157.2±53.0	160.2±42.2	161.2±40.8	157.7±56.6
	Latency trial 2 (seconds)	179.1±3.5	179.6±1.4	173.0±28.2	174.4±20.8

Trials to Criterion = Mean No. Trials per Group ± S.D.
Latency to Trial 1 = Mean Session 1 duration (seconds) per Group ± S.D.
Latency to Trial 2 = Mean Session 2 duration (seconds) per Group ± S.D.
Failed to Meet Criterion = Number of animals that received the shock but did not demonstrate acquisition.
Failed to Cross = Number of animals that received the shock but did not demonstrate acquisition.
Failed to Cross = Number of animals that never received the shock.
Values were not statistically different from control, $p \leq 0.05$.
Data obtained from page 55 of the study report.

Table KIIA 5.7.5/11b: Water Maze Performance at PND 60 (± 2 days) and Seven Days Later (mean ± S.D)

Session/Parameter		Dietary Level			
		Control	120 ppm	500 ppm	1200 ppm
Males					
Session 1 (Learning Phase)	Number of animals	16	16	16	16
	Trials to criterion (mean ± S.D.)	7.3±2.7	8.3±2.9	6.4±1.5	7.6±3.0
	Trial 1 – Errors (mean ± S.D.)	0.6±0.7	0.9±1.3	0.5±0.7	0.5±0.6
	Trial 1 – Duration (seconds) (mean ± S.D.)	18.3±16.7	19.5±13.8	17.7±16.2	16.7±8.4
	Trial 2 – Errors (mean ± S.D.)	0.7±0.9	0.8±0.7	0.5±0.5	0.6±0.9
	Trial 2 – Duration (seconds) (mean ± S.D.)	16.6±18.4	19.6±12.3	17.2±13.6	12.3±9.8
	Failed to meet criterion	1 (6%)	1 (6%)	0 (0%)	1 (6%)
Session 2 (Retention Phase)	Number of animals	15	15	16	15
	Trials to criterion (mean ± S.D.)	5.1±0.3	6.0±1.8	5.3±0.6	5.4±0.6
	Trial 1 – Errors (mean ± S.D.)	0.1±0.3	0.3±0.6	0.3±0.6	0.7±1.6
	Trial 1 – Duration (seconds) (mean ± S.D.)	4.7±3.0	6.2±5.1	7.1±5.9	8.7±9.9
	Trial 2 – Errors (mean ± S.D.)	0.0±0.0	0.3±0.5	0.1±0.5	0.1±0.5
	Trial 2 – Duration (seconds) (mean ± S.D.)	2.5±1.0	5.7±4.9	3.6±4.7	3.0±3.7
Females					
Session 1 (Learning Phase)	Number of animals	16	16	16	16
	Trials to criterion (mean ± S.D.)	8.0±2.9	7.9±3.9	7.6±2.3	7.1±2.0
	Trial 1 – Errors (mean ± S.D.)	0.4±0.5	1.0±1.8	0.7±0.9	0.5±0.6
	Trial 1 – Duration (seconds) (mean ± S.D.)	15.3±9.2	18.6±14.0	16.3±12.8	14.6±7.6
	Trial 2 – Errors (mean ± S.D.)	0.5±0.7	0.3±0.6	0.8±1.0	0.6±0.7
	Trial 2 – Duration (seconds) (mean ± S.D.)	13.1±8.2	11.3±15.1	16.0±14.9	12.8±4.4
	Failed to meet criterion	1 (6%)	3 (19%)	0 (0%)	0 (0%)
Session 2 (Retention Phase)	Number of animals	14	13	16	16
	Trials to criterion (mean ± S.D.)	6.2±1.4	6.9±2.9	5.7±1.7	5.9±1.3
	Trial 1 – Errors (mean ± S.D.)	0.3±0.5	0.5±1.0	0.3±0.8	0.6±1.2
	Trial 1 – Duration (seconds) (mean ± S.D.)	7.3±4.5	10.2±8.7	7.2±7.7	8.4±8.0
	Trial 2 – Errors (mean ± S.D.)	0.3±0.6	0.0±0.0	0.1±0.3	0.3±0.8
	Trial 2 – Duration (seconds) (mean ± S.D.)	5.4±4.3	3.8±2.2	5.1±4.8	5.1±4.4

Values for rats that failed to learn during Session 1 were not included in means for Session 2.
Values were not statistically significantly different from control, $p \leq 0.05$.
Data obtained from pages 55-56 of the study report.

3. Body weight, body weight gain, and food consumption

At 1200 ppm, bodyweight gain over PND 4 to 17 was statistically significantly ($p \leq 0.05$) lower in males (7% decrease) and was lower in females (6% decrease) but without statistical significance (*i.e*, $p > 0.05$). At this dose level, bodyweight gain over PND 11 to 17 was statistically decreased in both males (10% decrease) and females (7% decrease). Similar non-statistical trends in bodyweight gain were seen for both sexes over PND 4-21 and PND 11-21 (5% to 6% decreases). The study author regarded these effects to be test substance related. Bodyweight gain was unaffected in the 120 and 500 ppm groups (Table KIIA 5.7.5/11).

Bodyweight was non-statistically (i.e., p was >0.05) lower in males and females at 1200 ppm on PND 17 and 21 (4 to 4.5% decreases) relative to controls. The finding was regarded by the study authors to be test substance based on dose dependency, presence at multiple time points, and similar magnitude to lower dam bodyweights during lactation. There was no effect on offspring bodyweights at 120 or 500 ppm. There were was no effect on postweaning pup bodyweights (Table KIIA 5.7.5/12).

Overall the US EPA secondary reviewer considered the slight body weight decrease in the pups and associated body weight gain decease were not adverse.

Table KIIA 5.7.5/11: Preweaning pup bodyweights and bodyweight change (g)

PND	Dose (ppm) [N=23]							
	0	120	500	1200	0	120	500	1200
	M				F			
Preweaning pup bodyweight (g) [Mean (± S.E.)]								
0	5.9±0.13	5.9±0.10 (+0.0%)	6.0±0.10 (+1.7%)	6.0±0.10 (+1.7%)	5.6±0.11	5.7±0.10 (+1.8%)	5.7±0.08 (+1.8%)	5.7±0.11 (+1.8%)
4(pre cull)	9.9±0.25	9.8±0.20 (-1.0%)	9.9±0.24 (+0%)	10.0±0.26 (+1.0%)	9.5±0.23	9.5±0.20 (+0%)	9.4±0.21 (-1.1%)	9.5±0.28 (+0%)
4 (post)	10.0±0.25	9.8±0.20 (-2.0%)	9.9±0.24 (-1.0%)	9.9±0.26 (-1.0%)	9.5±0.24	9.5±0.20 (+0%)	9.4±0.21 (-1.1%)	9.4±0.28 (-1.1%)
11	25.4±0.48	24.9±0.46 (-2.0%)	25.7±0.44 (+1.2%)	24.5±0.49 (-3.5%)	24.5±0.44	24.3±0.46 (-0.8%)	24.6±0.42 (+0.4%)	23.7±0.47 (-3.3%)
17	38.9±0.65	37.9±0.55 (-2.6%)	39.2±0.56 (+0.8%)	36.8±0.60 (-5.4%)	37.5±0.56	37.0±0.59 (-1.3%)	37.6±0.47 (+0.3%)	35.8±0.54 (-4.5%)
21	47.9±1.04	48.4±0.85 (+1.0%)	47.6±0.66 (-0.6%)	45.6±1.02 (-4.8%)	46.3±1.01	47.2±0.96 (+1.9%)	45.4±0.65 (-1.9%)	44.4±0.83 (-4.1%)
Bodyweight change (g) over PND 4 to 21 [Mean (± S.E.)]								
4 to 17	28.9±0.56	28.1±0.45 (-2.8%)	29.3±0.41 (+1.4%)	**26.9±0.51*** **(-6.9%)**	27.9±0.50	27.5±0.48 (-1.4%)	28.2±0.33 (+1.1%)	26.3±0.43 (-5.7%)
4 to 21	37.9±0.88	38.6±0.73 (+1.8%)	37.7±0.57 (-0.5%)	35.7±0.87 (-5.8%)	36.8±0.88	37.7±0.82 (+2.4%)	36.0±0.56 (-2.2%)	34.9±0.68 (-5.2%)
11 to 17	13.6±0.31	13.0±0.20 (-4.4%)	13.5±0.23 (-0.7%)	**12.3±0.25** **(-9.6%)**	13.0±0.29	12.7±0.22 (-2.3%)	13.0±0.21 (+0%)	**12.1±0.18*** **(-6.9%)**
11 to 21	22.5±0.75	23.5±0.47 (+4.4%)	22.0±0.52 (-2.2%)	21.1±0.68 (-6.2%)	21.9±0.77	22.9±0.56 (+4.6%)	20.8±0.51 (-5.0%)	20.7±0.58 (-5.5%)

*denotes statistically significant at the * $p \leq 0.05$ or ** $p \leq 0.01$ level. No differences in absolute bodyweights were statistically significant. Data excerpted from page 45 of the study report, except for per cent change from control, which was calculated by the evaluator using Microsoft Excel.

Table KIIA 5.7.5/12: Postweaning Pup Body Weights (g)

PND	Dose (ppm) [N=23]							
	0	120	500	1200	0	120	500	1200
	M				F			
28	82.5±9.5	81.6±7.7	81.9±7.7	78.6±9.2	78.5±8.2	80.2±7.2	78.8±6.8	77.5±7.4
35	129.9±11.9	129.2±11.2	129.1±11.8	125.2±12.1	114.1±10.2	117.4±8.4	116.1±6.5	114.2±8.8
42	177.3±14.7	176.9±12.3	177.5±13.1	171.2±13.8	138.3±11.7	141.3±8.4	140.8±7.1	138.6±9.9
49	220.9±17.8	218.7±14.6	222.3±16.3	214.8±16.7	158.5±10.6	158.8±8.9	156.4±8.6	155.6±11.7
56	267.9±21.5	266.0±16.1	269.8±19.4	260.9±19.5	176.8±11.7	176.4±9.9	176.6±8.6	176.0±11.6
63	305.3±23.6	303.2±17.4	303.7±23.4	292.5±23.7	190.7±11.5	188.7±9.4	189.6±9.5	189.9±12.7
70	333.6±25.8	330.2±17.7	332.7±24.1	322.0±24.3	204.5±12.8	200.9±10.1	202.4±9.9	201.3±13.6

No differences were statistically significant at the $p \leq 0.05$ level. Data excerpted from page 46 of the study report.

4. Developmental landmarks

There were no adverse test substance-related effects on litter data endpoints during the course of this study (Table KIIA 5.7.5/13). The ages for onset of balanopreputial separation, vaginal patency, and surface righting, and pupil constriction in response to light at PND 21 were all unaffected by treatment.

Table KIIA 5.7.5/13: Mean (± S.E.) Developmental landmarks (days)

Parameter	Dose (ppm)			
	0	**120**	**500**	**1200**
Number of Litters (M/F)	23/23	23/23	23/23	23/23
Balanopreputial Separation	42.7±0.35	44.2±0.58	42.9±0.33	43.2±0.29
Vaginal Opening	32.3±0.51	33.9±0.85	33.4±0.65	32.3±0.44
Surface Righting	5.2±0.17	5.2±0.14	5.2±0.17	5.1±0.21
Pupil Constriction	21.0±0.00	21.0±0.00	21.0±0.00	21.0±0.00

No findings were statistically significant at the $p \leq 0.05$ level. Data excerpted from page 46 of the study report, except for per cent change from control, which was calculated by the evaluator using Microsoft Excel.

4. Ophthalmology

Ophthalmological findings were unremarkable in all groups.

5. Gross pathology

Gross necropsy findings were unremarkable, with a low incidence of spontaneous abnormalities seen in all groups including controls.

6. Organ weights

Organ weight findings in offspring were confined to a statistically significant decrease in absolute, mean non-perfused brain weight in males at 1200 ppm compared to controls (Table KIIA 5.7.5/14). The study author attributed this finding to the corresponding decreases in bodyweights in this group. As the brain is highly resistant to weight decreases resulting from generalized lower bodyweight gain or bodyweight loss, the evaluator disagrees that this is a basis to discount the finding. However, in the absence of any dose dependency, and noting the lack of a relative brain weight change compared with controls, the evaluator considers the finding to be unrelated to the test substance.

Table KIIA 5.7.5/14: Mean (± S.D.) fresh brain weight (g) on PND 70 to 80 (males)

Parameter	Male dosing group (ppm) [N=10]			
	0	**120**	**500**	**1200**
Terminal body weight (g)	334.6 ± 24.2	334.7 ± 24.0 (+0%)	341.7 ± 30.5 (+2%)	318.7 ± 35.0 (-5%)
Abs brain weight	1.956±0.090	1.985±0.034 **(+1%)**	1.949±0.070 **(+0%)**	**1.868±0.085*** **(-4%)**
Rel brain weight (to BW)	0.587±0.046	0.596±0.041 **(+2%)**	0.574±0.046 **(-2%)**	0.591±0.049 **(+1%)**

* denotes statistically significant at the $p \leq 0.05$ level. Data excerpted from page 59 of the study report, except for per cent change from control, which was calculated by the evaluator using Microsoft Excel.

7. Brain morphometry – macroscopic

At both PND 21 and PND 75, there were no differences in anterior/posterior cerebrum or cerebellum measurements in any groups.

8. Brain morphometry – sections (neuropathology)

Changes in magnitude of up to 5% (hippocampal gyrus for females) in brain structure lengths were seen in both sexes at 1200 ppm at PND 21 relative to controls, but were without statistical significance and were not regarded by the study authors to be test substance related (Table KIIA 5.7.5/15). Therefore, analyses of these parameters were not undertaken in the 120 and 500 ppm dosing groups. The evaluator agreed with the study author's conclusion that there were no morphometry differences in brain sections associated with treatment.

Table KIIA 5.7.5/15**: Mean brain measurements (mm)**

Parameter	M		F	
	dosing group (ppm) [N=8 to 10]			
	0	1200	0	1200
Frontal Cortex				
– PND 21	1.29±0.01	1.26±0.01 (-2.3%)	1.26±0.004	1.22±0.005 (-3.2%)
– PND 75	1.28±0.007	1.25±0.025 (-2.3%)	1.20±0.004	1.18±0.005 (-1.7%)
Hippocampal gyrus				
– PND 21	1.18±0.04	1.15±0.01 (-2.5%)	1.21±0.019	1.15±0.008 (-5.0%)
– PND 75	1.33±0.006	1.38±0.013 (+3.8%)	1.30±0.007	1.26±0.013 (-3.1%)
Cerebellum				
– PND 21	3.87±0.01	3.90±0.06 (+0.8%)	3.82±0.07	3.71±0.06 (-2.9%)
– PND 75	3.91±0.07	3.95±0.10 (+1.0%)	3.72±0.07	3.76±0.03 (+1.1%)

Data excerpted from pages 60 and 1059-1061 of the study report, except for per cent change from control, which was calculated by the evaluator using Microsoft Excel.

9. Brain and peripheral nerve histopathology

Degeneration of individual nerve fibers noted in gasserian ganglion (at 1200 ppm females) and left tibial nerve (at 1200 ppm males), each in 1/10 animals at PND 70, were regarded to be incidental findings and to be unrelated to the test substance.

III. CONCLUSION

Under the conditions of this study, for dams, there were no test substance-related effects on maternal clinical signs, FOB parameters, mortality, or reproductive outcomes. For offspring, there were no test substance related effects on clinical signs, mortality, acquisition and retention parameters for the passive avoidance and water maze tasks, sexual maturation, surface righting, pupil reflex or other ophthalmology parameters, necropsy findings, organ weights, macroscopic or histological brain morphometry, or brain or peripheral nerve histopathology.

During gestation, a decrease in body weight was seen (↓7%) in 1200 ppm dams and associated decreases in body weight gain were present. At lactation period a decrease of (4% to 6 %) in body weight was also found in1200 ppm dams. A slight decrease in food consumption was found in the dames at various times during the gestation and lactation periods (ranging from 1% to 8%). However, the changes were slight and not considered as adverse.

For pups, a non-statistically significant increase in both motor and locomotor activity was seen in males at 1200 ppm on PND 13 relative to concurrent controls. On PND 60, startle amplitude for all

50 trials was statistically significantly ($p \leq 0.05$) increased by approx. 2-fold in females at 1200 ppm relative to controls. The increase was above the historical control.

There was no evidence of treatment-related changes in brain morphometry or peripheral nerve histopathology.

Under the conditions of this study, no adverse effects were found in the dam at the highest dose tested; the maternal NOAEL was established at 1200 ppm (102 mg/kg/day, highest dose tested). The offspring NOAEL was 500 ppm (42.4 mg/kg/day); LOAEL was 1200 ppm (102 mg/kg/day) based on statistically significant increased in startle response amplitude.

This study is reliable (acceptable/guideline) and satisfies the requirements for a developmental neurotoxicity study in rats **(**OPPTS 870.6300; OECD 426).

Primary review: Australia
Secondary review: US EPA

IIA 5.1.1/01 ADME Study in rats: [Pyridinylmethyl-^{14}C]BYI02960

Report: Klempner, A (2012) [Pyridinylmethyl-^{14}C]BYI02960 – Absorption, Distribution, Excretion, and Metabolism in the Rat. Bayer CropScience AG, Development - Environmental Safety, Metabolism/ADME and Environmental Fate, D-40789 Monheim am Rhein, Germany. Study No. M1824559-4. January. MRID 48844141. Unpublished.

Guidelines: US EPA 870.7485 (1998)
OECD TG 417 (2010)
JMAFF 12 Nousan 8147 (2000)
Deviations: None

GLP: Yes. Signed and dated GLP, Quality Assurance and Data Confidentiality statements were presented in the report.

Executive summary:

In a toxicokinetic and metabolism study (MRID 48844141), groups of 4 Wistar rats per sex per dose were administered by oral gavage a single dose of 2 or 200 mg/kg bw [Pyridinylmethyl-^{14}C]BYI02960 in 0.5% aqueous Tragacanth® and sacrificed by exsanguination under anaesthesia at 72 hours post dosing. An additional group of 4 male Wistar rats were administered by i.v. a single dose of [Pyridinylmethyl-^{14}C]BYI02960 (2 mg/kg bw) in the same vehicle and sacrificed at the same time post dosing. The test compound was radiolabelled with ^{14}C in the pyridinylmethyl bridge of the molecule. The metabolism and radioactivity which included the test compound and metabolites was determined in urine and faecal samples collected for the time range between dosing and sacrifice. Additionally, radioactivity in plasma micro samples was determined over the study period (17 time points). Total radioactivity was determined in excreta and in organs and tissues collected at sacrifice. Samples were analysed, and parent compound and metabolites identified, by radiochromatographic (HPLC, TLC) and spectroscopic (LCMS) methods.

Mean total radioactivity was nearly quantitative since approximately 91 to 103% of the administered dose was found in the excreta and in the bodies of animals at sacrifice.

[Pyridinylmethyl-^{14}C]BYI 02960 was nearly completely absorbed after oral administration, which was demonstrated by the high factor of bioavailability (F = 0.93) calculated from the plasma data of low dose tests after oral and i.v. administration, and that approximately 76 to 90% of the administered radioactivity were detected in the urine and in the bodies without GIT at sacrifice of male and female rats. The absorption started immediately after dosing as could be seen from the quick increase of radioactivity in plasma micro samples, with the peak plasma concentration (C_{max}) reached approximately 1 hour after administration in the low dose tests and within approximately 2 to 4 hours in the high dose tests.

The distribution of the radioactivity within the body was fast. From the maximum plasma level (C_{max}), the radioactivity level declined slowly down to approximately 50% of C_{max} after 4 – 8 hours in the low dose tests and after 8 – 24 hours in the high dose tests and down to low values around the LOQ in the low dose tests and to approximately 0.5% of C_{max} in the high dose tests by study termination (72 hours).

Excretion was rapid, mainly by the renal route and essentially complete by 72 hours post dosing. Female rats exhibited slightly higher renal excretion rates of approximately 86% and 90% of the administered dose compared to approximately 76% of the dose in males. The major part of the dose detected in urine (>84%) was excreted within 24 hours after treatment and was ongoing up to 72 hours. Faecal excretion accounted for approximately 23 – 26% of the total administered in males, and 7 – 10% in females. At the time of sacrifice, 72 hours after administration, the radioactive residues in organs and tissues were low, and only trace amounts of approx. <0.1 – 0.3% of the total administered dose was detected in the body and in the GIT. Most of the residues of the low dose tests were lower than 0.01 mg/kg sample. The highest concentrations of the low dose tests, but also at a low level of approximately ≤0.018 mg/kg sample were detected in blood cells, the GIT and in the eyes of female rats. The residues of the low and high dose tests were nearly proportional to the dose.

Parent compound, three major (BYI 02960-OH, BYI 02960-6-CNA and BYI 02960-hippuric acid) and five minor metabolites were isolated from urine and four of them identified by spectroscopic methods (LC-MS and 1H-NMR and 2D-NMR), HPLC and/or TLC co-chromatography or comparison. Identification rates were high in excreta, and amounted to 83% to 94% of the total administered radioactivity. Parent compound represented the predominant part of the radioactivity in urine of male and female rats. In faeces samples of male rats, the metabolite BYI 02960-OH was more prominent than the parent compound. Two metabolites, BYI 02960-6-CNA and BYI 02960-hippuric acid were also prominent metabolites in male rats but not in females. In total further 19 unknown metabolites were characterised by their chromatographic behaviour. All of them were detected at trace amounts of approximately <0.1% to 0.9% of the dose. The metabolic profiles in urine and faeces were very similar for both sexes but male rats exhibited a higher rate of metabolite formation compared to female animals. The metabolic transformation of BYI 02960 was principally oxidative and took place at least at 3 different structural positions of the test compound.

The principal metabolic reactions of [pyridinylmethyl-14C]BYI 02960 in rats were: (1) hydroxylation followed by conjugation with glucuronic acid to or with sulphate; (2) cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl; and cleavage of the molecule at the pyridinylmethyl bridge forming BYI 02960-6-CNA, which was further conjugated with glycine to BYI 02960-hippuric acid.

The results of the toxicokinetic and metabolism study were in good accordance with those obtained in other studies performed with different radiolabels: [Ethyl-1-^{14}C]BYI

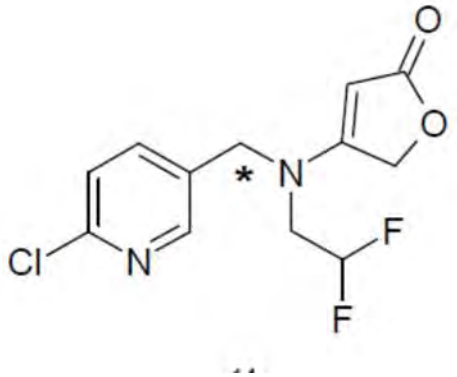
02960 (Weber, 2011a; Study No. M1824567-3, Koester, 2012a; Study No. M1824575-2) and [Furanone-4-^{14}C]BYI 02960 (Weber, 2011b; Study No.M1824560-6, Koester, 2012a; Study No. M1824574-1, Koester, 2011b, Study No. M1821760-5).

This study is fully reliable (acceptable/guideline) and satisfies the study requirements of a metabolism study (U.S. EPA 870.7485; OECD Section 4 Part 417 and Annex to Directive 87/302/EEC, Part B, Toxicokinetic; JMAFF 12 Nousan 8147).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI02960
   Batch #'s: BYI 02960-PU-02
   Purity: 99.4%
   Description: White powder
   CAS #: 951659-40-8
   Stability of test compound: Stated to be stable for two years if stored at 5±5ºC (assay date 27 Oct 2008).
2. Radiolabeled test material: [pyridinylmethyl-^{14}C]BYI02960
   Lot/Batch #: KATH 6264 (batch 1, tests 1 -4), KATH 6351 (batch 2, test 5).
   Chemical structure /positions of radiolabel:



* Denotes position of radiolabel

   Radio-chemical purity: [pyridinylmethyl-^{14}C]BYI02960: >99% (HPLC, UV), >99% (TLC)
   Specific activity: [pyridinylmethyl-^{14}C] 118.08 µCi/mg
   Description: Solid, dried in vacuum
   Stability of test compound: The positions of the radiolabels are considered to be stable.
3. Vehicle and/or positive control: 0.5% aqueous Tragacanth®
4. Test animals
   Species: Rat
   Strain: Wistar Hsd/Cpb: WU
   Age at dosing: 7 to 8 weeks (males), 9 to 10 weeks (females)

Weight at dosing:	195 to 242 g (males), 192 to 212 g (females)
Source:	Harlan Netherlands BV, Kreuzelweg 53, NL-5960 AD Horst, Netherlands.
Acclimation period:	Seven days in Makrolon® metabolism cages prior to dosing.
Diet:	Rat/mice maintenance long life diet No. 3883.0.15, (Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland), *ad libitum*, except for approximately 16 hours prior to, and for 8 hours after dosing.
Water:	Tap water, *ad libitum*.
Housing:	Individually in Makrolon® metabolism cages with wood floor shavings until termination. Cages permitted an 'almost' quantitative separate urine and faeces collection.

5. Environmental conditions

Temperature:	21 to 25°C
Humidity:	45 to 71%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

6. Preparation of the dosing solutions

Rats were dosed with [pyridinylmethyl-^{14}C]BYI02960. The [pyridinylmethyl-^{14}C]BYI02960 radiolabel was diluted with technical BYI02960. The dose vehicle 0.5% aqueous Tragacanth® was added using an ultrasonic bath and stored at 5ºC until use and kept homogenous by magnetic stirring bars and glass balls. Dose route, dose level, volume, and radiolabel data are presented in Table 1. The test compound was administered by a single oral gavage or i.v. dose.

Table 1. Dose Levels, Volumes, and Radiochemical Data

Test No.	Dose route	Administration suspension							
		Total prepared		Administered per rat					
		Amount (mg)	Volume (mL)	Amount (mg)	Volume (mL)	Radioactivity (dpm)	Target dose (mg/kg bw)	Actual dose (mg/kg bw)	Mean rat weight (g)
1	Oral	2.72	13.6	0.40	2	104, 767, 520	2	1.95	205
2		3.2	16.0	0.41	2	108, 408, 579	2	1.98	209
3		320.1	16.0	39.82	2	104, 377, 643	200	194.3	205
4		230.0	16.0	34.74	2	91, 81, 460	200	172.8	201
5	i.v.	5.6	7.0	0.39	0.5	103, 473, 27	2	1.70	232

Table excerpted from page 21 of the study report.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

   18 March 2009 to 12-January-2012

2. Main study

   The dose level was based on the specific radioactivity, stability of the dosing suspensions, and the fact that 2 to 5 mg/kg bw doses used as the low dose levels for ADME studies in rats by Weber (2011a/b, Study no's: M1824567-3; M1824560-6), and Koester (2011a, Study no. M1821837-0), had not produced any observable toxicity. The basis for choosing 200 mg/kg bw as the high dose was not explained, but the study authors stated that only minimal clinical signs of toxicity were observed at this dose. Animals were assigned to test groups according to Table 2.

Table 2. Study design

Group	No. of animals	Dose, route (mg/kg bw)	Sampling intervals or timepoint (hours)		
			Urine	Faeces	Plasma microsample
1	4 males	2, oral	0 – 4, 4 – 8, 8 – 12, 12 – 24, 24 – 48, 48 – 72	0 – 24, 24 – 48, 48 -72	10 min, 20 min, 40 min, 1, 1.5, 2, 3, 4, 6, 8, 24, 28, 32, 48, 52, 56, 72
2	4 females	2, oral	0 – 4, 4 – 8, 8 – 24, 24 – 48, 48 - 72	0 – 24, 24 – 48, 48 -72;	0 min, 20 min, 40 min, 1, 1.5, 2, 3, 4, 6, 8, 24, 28, 32, 48, 52, 56, 72;
3	4 males	200, oral	0 – 4, 4 – 8, 8 – 12, 12 – 24, 24 – 48, 48 - 72	0 – 24, 24 – 48, 48 -72	10 min, 20 min, 40 min, 1, 1.5, 2, 3, 4, 6, 8, 24, 28, 32, 48, 52, 56, 72
4	4 females	200, oral	0 – 4, 4 – 8, 8 – 24, 24 – 48, 48 - 72	0 – 24, 24 – 48, 48 -72	10 min, 20 min, 40 min, 1, 1.5, 2, 3, 4, 6, 8, 24, 28, 32, 48, 52, 56, 72
5	4 males	2, i.v.	0 – 4, 4 – 8, 8 – 24, 24 – 48, 48 - 72	0 – 24, 24 – 48, 48 -72	10 min, 20 min, 40 min, 1, 1.5, 2, 3, 4, 6, 8, 24, 28, 32, 48, 52, 56, 72

Note: Time of sacrifice was 72 hours post-dosing for all groups.

3. Plasma Kinetics Following Dosing (10 min – 72 hours)

In-life blood samples from individual animals were obtained by pressing a heparin coated capillary into a small cut in the tail vein which was subsequently closed by tape. Blood samples were separated afterwards into plasma and blood cells by centrifugation. At 72 hours post-dosing animals in each group were sacrificed by transection of the cervical vessels and exsanguination under Pentobarbital-Na anaesthesia. Oozed out blood samples from individual animals was collected in heparinised test tubes and separated afterwards into plasma and blood cells by centrifugation. After weighing, aliquots from the whole plasma sample were taken for determination of total radioactivity by liquid scintillation counting (LSC).

4. Tissue Distribution at sacrifice
Tissues and carcass were analyzed to determine ^{14}C tissue distribution by combustion/LSC. The following tissues were collected at sacrifice from test animals (all test groups unless indicated otherwise):

Blood (plasma & RBC)	Liver	Femur
Kidney	Leg muscle	Heart
Lung	Testes (Groups 1, 3, 5)	Ovaries (Groups 2 & 4)
Uterus (Groups 2 & 4)	Bone femur	Brain
Spleen	Adrenals	Thyroid
G.I. tract and contents	Skin	Harderian gland
Eyes	Perirenal fat	

5. Quantitation of Radioactive Residues

Radioactive residues of all individual animals were measured in plasma, urine (including cage rinse i.e. funnel rinse from the Makrolon® metabolism cage) and faeces samples, and tissues. The measurement of the radioactivity in liquid samples was carried out by LSC. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

6. Calculation of toxicokinetic parameters and bioavailablity

Standard toxicokinetic parameters for plasma were calculated, such as.

$AUC_{0-\infty}$	Area under the plasma radioactivity concentration-time curve extrapolated from time 0 to infinity
$t_{1/2\ a}$	half-life of the absorption
$t_{1/2\ e}$	half-life of the elimination (terminal half-life)
C_{max}	maximum radioactivity concentration in plasma after single (first) dose administration
t_{max}	time at which the highest radioactivity concentration occurs in plasma after a single dose
MRT_{tot}	mean residence time of the total radioactivity in the measurement compartments, usually extrapolated from time 0 to infinity
MRT_{abs}	mean residence time of the absorption
MRT_{disp}	mean residence time of the disposition

The evaluation was performed with dose normalised values for the plasma concentrations for better comparison of the low dose tests among themselves and to the

high dose tests. A standard two compartment disposition model was used for curve fitting computation.

Bioavailability was calculated by incorporating dose normalised AUC values for orally dosed animals (Groups 1 to 4) and those for intravenously dosed animals (Group 5) in the following equation:

$$F = (AUC_{exp} / AUC_{i.v.}) \times (Dose_{i.v.} / Dose_{exp})$$

F	bioavailability
AUC_{exp}	area under the plasma concentration-time curve after oral administration.
$AUC_{i.v.}$	area under the plasma concentration-time curve after intravenous administration.
$Dose_{i.v.}$	administered dose of oral low dose test [mg/kg bw]
$Dose_{exp}$	administered dose of intravenous low dose test [mg/kg bw]

7. Quantification and Identification of Metabolites

For metabolic profiling, the urine and faecal samples were pooled across individual animals for a given collection interval for each sex. Liquid chromatography (LC)/mass spectrometry (MS) methods were employed for identification and quantification of the metabolites in each sample. Parent compounds and metabolites were identified by:

- spectroscopic evidence (LC-MS and high resolution LC-MS analysis, and 1H-NMR and 2D-NMR analysis) following isolation and purification from urine pool 0 – 48h of test 4 (high dose test with females),
- HPLC and TLC co-chromatography of urine samples or isolated peaks with the isolated and identified metabolites and with non-radiolabelled reference compounds,
- HPLC co-chromatography with egg extract and the isolated metabolite (BYI02960-OH-SA) from egg extract of the laying hen metabolism study performed with [pyridinylmethyl-14C]BYI 02960,
- enzyme treatment of isomer 1 and isomer 3 of BYI 02960-OH-gluA with glucuronidase / arylsulfatase along with comparison of the HPLC profiles of the metabolites before and after cleavage to the profiles of urine, faeces extract and reference compounds,
- comparison of the HPLC profiles of urine pools of the several time intervals of test 1 among themselves and with the ones of all other tests,
- comparison of the HPLC profiles of all faeces extracts among themselves and with the profiles of urine, and
- comparison of urine profiles of the present study with the ones of the rat organ metabolism study and with urine of the rat ADME study both performed with

[furanone-4-14C]BYI 02960 (Koester., 2012b; Study No. M1824574-1; Weber, 2011b; Study No. M1824560-6).

Other peaks or peak groups additionally detected in the HPLC-profiles were designated as "unknown". They were characterised by their behaviour during extraction and clean-up and the retention times in the HPLC-chromatograms.

Parent compound and metabolites were quantified in the original urine samples and faeces extracts by integration of the ^{14}C-signals in the HPLC-chromatograms.

II. RESULTS AND DISCUSSION

A. STORAGE STABILITY

Using HPLC profiling, the test substance and major metabolites were found to be stable in urine and faeces extracts over a time interval of at least two months.

B. MAIN STUDY

Clinical Observation

Inactivity after dosing was observed in an unspecified number of females following oral administration of 200 mg/kg over an unspecified interval, with the finding fully resolving by six hours post dosing.

Recovery

Mean total radioactivity recoveries for the orally dosing groups ranged from approximately 97% to 103% of the administered dose (groups 1 – 4), and was slightly lower at approximately 91% of the administered dose for the intravenous dosing group (group 5). For all dose groups, the majority of radioactivity was recovered from the urine, and together with the moderate recovery from faeces, excreta accounted for almost all of the recovered radioactivity (Table 3).

Table 3. Excretion and radioactive residues following a single oral or i.v. dosing of [pyridinylmethyl-^{14}C]BYI02960 to rats (Per cent of radioactive dose administered)

	2 mg/kg bw			**200 mg/kg bw**	
	M, oral (group 1)	**M, i.v. (group 5)**	**F, oral (group 2)**	**M, oral (group 3)**	**F, oral (group 4)**
	Mean +CV[%]	**Mean +CV[%]**	**Mean +CV[%]**	**Mean +CV[%]**	**Mean +CV[%]**
Faeces	23.09±4.31	14.64±22.29	7.49±19.61	26.14±8.01	10.32±13.70
Urine	75.45±2.25	76.24±3.58	90.07±2.31	76.26±2.91	85.95±4.31
Sum of excreta	98.55±1.02	90.88±1.94	97.56±1.48	102.40±0.51	96.26±4.70
Body excl GIT	0.119±13.20	0.141±7.67	0.064±9.18	0.128±1.21	0.241±41.94
GIT	0.069±37.47	0.086±14.89	0.010±24.81	0.086±41.55	0.064±44.24
Total Body	0.188±21.58	0.227±10.28	0.074±10.95	0.214±16.47	0.306±36.59
Balance	**98.73±1.03**	**91.11±1.94**	**97.63±1.49**	**102.60±0.52**	**96.57±4.59**

Data excerpted from pages 180, 185, 190, 195, and 200 of the study report.

Absorption

The pharmacokinetic results indicated that absorption occurred immediately following low (2 mg/kg bw) and high (200 mg/kg bw) administration of a single oral gavage dose.

Gastrointestinal absorption of the test substance was at least 76% in males administered 2 and 200 mg/kg bw, and 90% in females administered 2 mg/kg bw and 200 mg/kg bw, as >76% and 90% of the total dose administered was detected in urines and in the carcass excluding GIT respectively. The peak plasma concentration (C_{max}) was reached approximately 1 hour in males and females administered the low (2 mg/kg bw) dose, and 2 and 4 hours in males and females administered the high (200 mg/kg bw) dose respectively (Table 4). Additionally, it can be seen that plasma values in oral low (2 mg/kg bw) dose groups were approximately equivalent to values following i.v. dosing (2 mg/kg bw) at 40 minutes to 1 hour post dosing.

Table 4. Summary of time course of mean radioactivity measured in the plasma following a single oral or i.v. dose of [pyridinylmethyl-14C]BYI02960 to male or females

	Dose (mg/kg bw)				
	2			**200**	
	M, oral (group 1)	**M, i.v. (group 5)**	**F, oral (group 2)**	**M, oral (group 3)**	**F, oral (group 4)**
Time (hours)	**Mean mg a.s. equiv. /kg sample**				
0.17	0.4465	1.6070	0.5287	32.5100	30.9100
0.33	1.0380	1.6970	1.2010	66.3400	57.2100
0.67	1.5690	1.7670	1.7240	88.9400	74.9300
1	1.7130	1.7060	1.8540	94.3200	84.4500
1.5	1.6740	1.5570	1.7920	96.0300	87.7900
2	1.5500	1.3850	1.7630	96.8600	92.7700
3	1.3410	1.1180	1.6100	95.6200	98.3200
4	1.1990	0.8976	1.4500	93.3100	99.9900
6	0.7990	0.6250	1.1350	80.6500	91.7700
8	0.5281	0.4357	0.8420	69.2600	81.9800
24	0.0200	0.0193	0.0354	6.4460	22.4000
28	0.0134	0.0157	0.0228	3.3800	14.8300
32	0.0100	0.0103	0.0157	2.1660	10.1800
48	NC	0.0062	0.0052	0.6626	1.4800
52	NC	0.0053	NC	0.6165	1.2700
56	NC	0.0038	NC	0.5762	0.9593
72	NC	0.0044	NC	0.3602	0.5697

NC – denotes mean values not calculated because more than one third of the individual values used to derive the mean were below the LOQ

Data excerpted from page 59 of the study report.

Following oral dosing, the plasma concentrations declined to approximately 50% of Cmax within 4 – 8 hours and to approximately 1 – 2% of Cmax within 24 hours in the

low (2 mg/kg bw) dose groups in male and female rats. In the high dose (200 mg/kg bw) dose groups in male and female rats, the decline phases were slightly delayed, and the decline to approximately 50% of Cmax reached within 8 to 24 hours and to approximately 1 – 2% after 48 hours, respectively. The concentrations declined further steadily in the low and high dose groups. At the time of sacrifice, 72 hours after administration, the plasma concentrations were below or around the LOQ for male and female animals of the low (2 mg/kg bw) dose groups and at approx. 0.5% of Cmax in the high (200 mg/kg bw) dose group.

Biokinetic modelling of mean values between 0 to 32 hours was undertaken for all dose groups (as about 32 – 48 hours post administration concentrations ion plasma of the low dose groups were below or close to the LOQ), using a two compartment model. For better curve fitting, the values of the low dose tests with male rats only were evaluated using the 1/Y (group 1) or the 1/Y2 (group 5) individual weighting functions (1 and 32 hours values for group 1 and 4 and 6 hours values for group 2). The corresponding concentration-time curve of the modelled plasma data is shown in Figure 1 and Figure 2.

Figure 1: Comparison of dose normalised plasma curves of low dose (oral and i.v.)groups

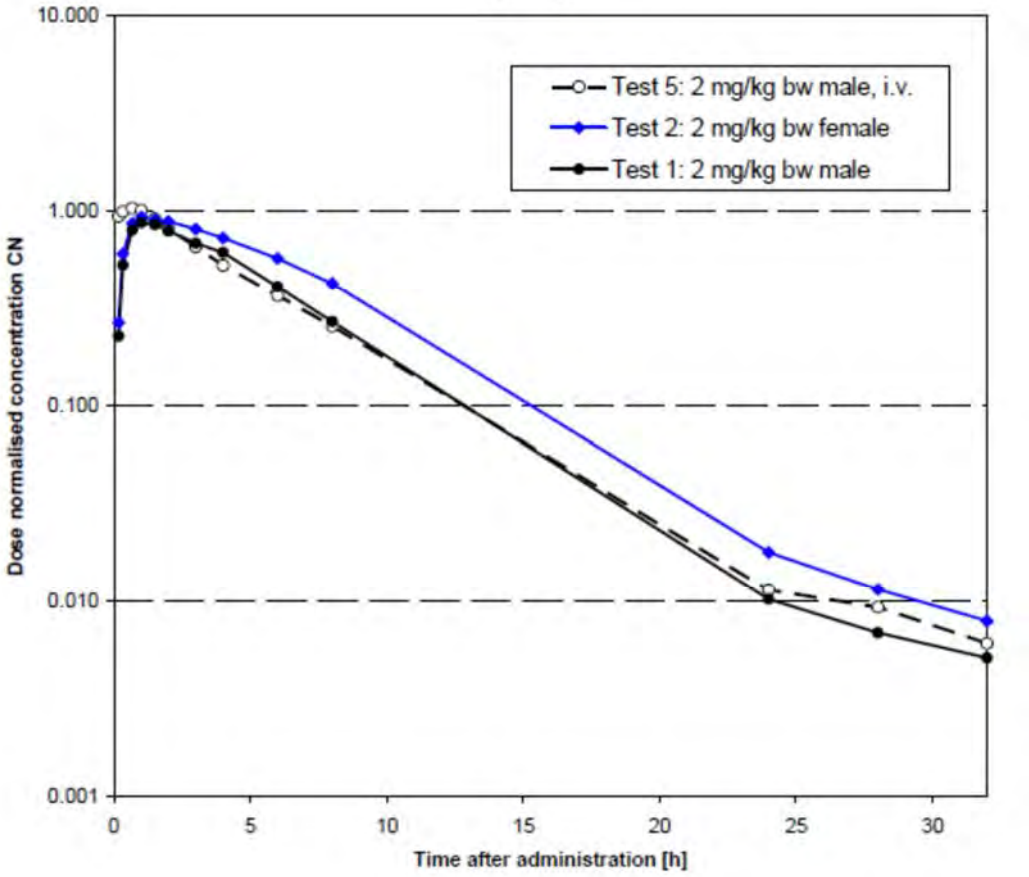


Figure excerpted from page 82 of the study report.

The plasma curves of male rats of the low dose groups after oral and i.v. administration were comparable. The plasma curve of the low dose females was slightly higher than that of the males but still with a similar curve shape.

Figure 2: Comparison of dose normalised plasma curves of low and high dose oral groups

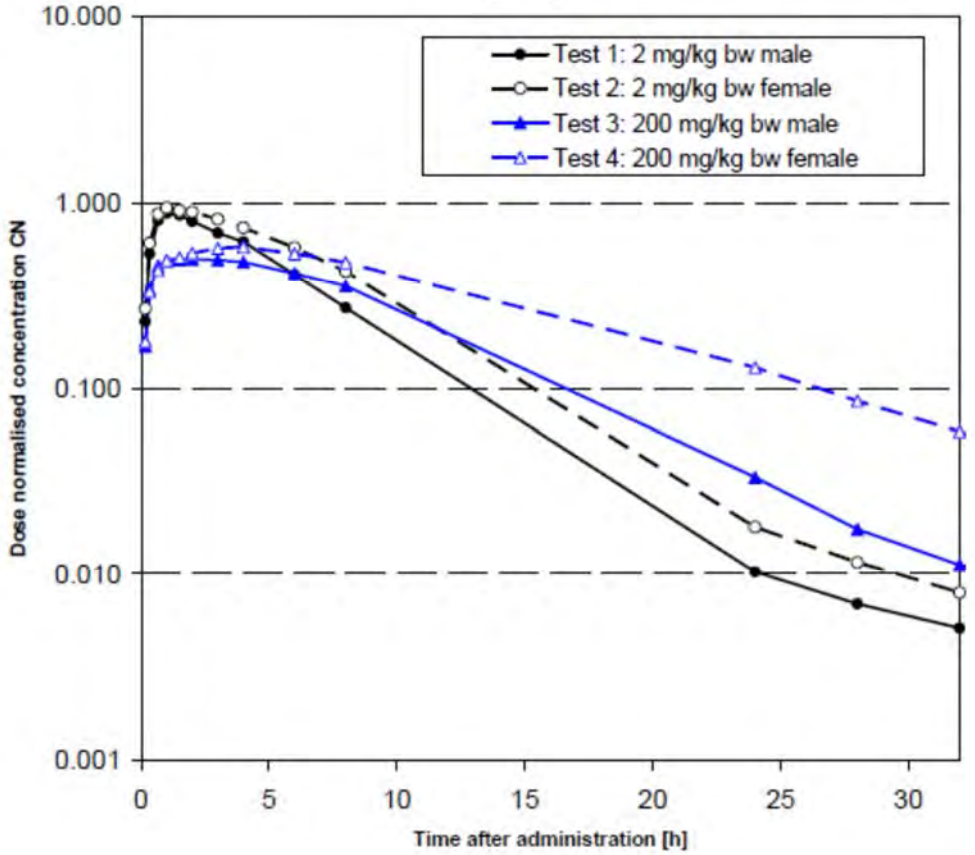


Figure excerpted from page 83 of the study report.

The plasma curves of the high dose groups showed a broader maximum for males and a distinctly broader maximum for females when compared with the low dose groups due to ongoing GIT absorption of the test compound.

The oral biokinetic parameters were comparable in the three low dose tests. The AUC extrapolated from 0 to infinity for females of the low dose (2 nmg/kg bw) group was approximately 1.3 times higher than for males, which is related to the slightly broader peak maximum of the plasma curve of female rats. In the high dose (200 mg/kg bw) groups, the AUC value for males was proportional to the dose. For females, the AUC

value of the high dose (200 mg/kg bw) group was approximately 1.2 times higher than for the low dose (2 mg/kg bw) group with females and approximately 1.6 times higher than for the high dose (200 mg/kg bw) group with males. According to the broader maximum of the plasma curve of female rats in the high dose (200 mg/kg bw) group, C_{max} was reached later than in males (approx. 4 hours compared to 2 hours). The biokinetic model confirmed a fast absorption phase in all tests with short half-lives of a maximum of approximately 0.2 hours followed by a short elimination phase with half-lives of approximately 3 – 4 hours, with females in the high dose (200 mg/kg bw) group showing a longer elimination phase with a half-life of about 8 hours. The mean residence times in the low dose tests were in the range of approximately 6 – 8 hours. The mean residence times were slightly longer in the high dose tests, approximately 9 hours for males and 13 hours for females. A summary of the pharmacokinetic pararmeters is shown in Table 5.

Table 5: Pharmacokinetic parameters and calculation of the bioavailability of [pyridinylmethyl-14C]BYI02960 after oral dosing to male and females, and after i.v. dosing to males

Test Group	**1**	**5**	**2**	**3**	**4**
Sex	**M**	**F**	**F**	**M**	**F**
Dose (mg/kg bw)	**2 (oral)**	**2 (*i.v.*)**	**2 (oral)**	**200 (oral)**	**200 (oral)**
Tmax measured [h]	1.0	0.67	1.0	2.0	4.0
Tmax calculated [h]	1.13	0.38	1.15	2.23	3.35
Cmax measured	0.878	1.040	0.937	0.497	0.578
Cmax calculated	0.880	1.040	0.929	0.500	0.582
$T_{1/2\ abs}$ [h]	0.21	0.06	0.17	0.13	0.17
$T_{1/2\ elim}$ [h]	3.9	3.8	3.0	3.6	8.1
$AUC_{0-\infty}$ [mg/L/h]	**6.10**	**6.55**	**7.96**	**6.16**	**9.73**
MRT_{tot} [h]	6.07	5.71	6.69	8.70	13.10

Data excerpted from page 61 of the report.

A bioavailability factor (F) of 0.93 was obtained demonstrating that the orally administered test compound was almost completely bioavailable.

Distribution

A mean of approximately 0.1 - 0.3% of the total administered dose was detected in the body at the time of sacrifice 72 hours after oral and i.v. dosing, of which only 0.01 - 0.09% was in the GIT. Mean residual concentrations in tissues were low in the low dose (2 mg/kg bw) group but indicative of wide distribution, and ranged from 0.0007 (muscle leg, females administered oral dose of 2 mg/kg bw) to 0.158 mg/kg sample (blood cells, males administered i.v. dose of 2 mg/kg bw). Levels for most organs and tissues were very similar in the low dose (2 mg/kg bw) groups and in the range of approximately 0.001 to 0.007 mg/kg sample (Table 6). Mean residual concentrations in tissues in the high dose (200 mg/kg bw) groups were were nearly proportional to the dose and ranged from 0.0859 (brain, males administered oral dose of 200 mg/kg bw) to 2.345 mg/kg sample ((blood cells, males administered oral dose of 200 mg/kg bw). For most organs and tissues, the residue levels in the high dose (200 mg/kg bw) groups were slightly higher in males compared to females (Table 6).

Table 6: Radioactive residues in organs and tissues at sacrifice following single oral or i.v. dosing of [pyridinylmethyl-^{14}C]BYI02960 to rats (Equivalent concentration [mg a.s. equiv. /kg sample])

Organ/tissue	Dose (mg/kg bw)				
	2			200	
	M, oral	M, i.v.	F, oral	M, oral	F, oral
Blood cells	0.0175	0.0158	0.0067	2.3450	1.5770
Plasma	0.0020	0.0025	0.0013	0.2989	0.2963
Carcass	0.0021	0.0020	0.0011	0.1794	0.2377
Heart	0.0024	0.0023	NC	0.2643	0.3328
Brain	0.0008	0.0008	NC	0.0859	0.0922
Kidneys	0.0064	0.0067	0.0033	0.7975	0.6691
Liver	0.0068	0.0063	0.0034	0.8741	0.7720
GIT	0.0141	0.0167	0.0019	1.7290	1.1450
Testes	0.0008	0.0011	---	0.1020	---
Ovaries	---	---	NC	---	0.2880
Uterus	---	---	0.0016	---	0.4599
Adrenal gland	0.0048	0.0045	0.0032	0.4436	0.5606
Harderian gland	0.0050	0.0034	0.0022	0.4101	0.7218
Thyroid gland	NC	NC	NC	NC	NC.
Spleen	0.0030	0.0032	0.0017	0.3586	0.3398
Lung	0.0060	0.0054	0.0035	0.6648	0.5663
Eyes	0.0064	0.0066	0.0133	0.5996	1.3430
Skin	0.0018	0.0024	0.0014	0.2354	0.9710
Bone femur	NC	0.0024	0.0023	0.1771	0.2794
Fat perirenal	0.0018	0.0030	NC	NC	0.1245
leg muscle	0.0008	0.0012	0.0007	0.1206	0.2098

NC: not calculated

Data excerpted from page 62 of the study report.

Excretion

Excretion was rapid and essentially complete by 72 hours post dosing with a mean of approximately 98% and 99% of total administered dose being excreted by this time point in males and females administered 2 mg/kg bw by oral gavage respectively, and approximately 102 and 96% in males and females administered 200 mg/kg bw by oral gavage respectively. In males administered 2 mg/kg bw i.v., approximately 91% of total administered dose was excreted by 72 hours post dosing. The major route of excretion was urine, accounting for approximately 76% and 90% in males and females administered 2 mg/kg bw by oral gavage respectively, and approximately 76% and 86% in males and females administered 200 mg/kg bw by oral gavage respectively (and approximately 90% in males administered 2 mg/kg bw by i.v.). Faecal excretion accounted for approximately 23% and 7% of the total administered dose in males and females administered 2 mg/kg bw by oral gavage, and 26% and 10% in males and females administered 200 mg/kg bw by oral gavage respectively (and approximately 15% in males administered 2 mg/kg bw by i.v.). For both urinary and faecal excretion the major part of the radioactivity was excreted within 24 hours (Table 7).

Table 7: Time course of excretion of radioactive residues via urine and faeces following dosing of [pyridinylmethyl-14C]BYI02960 to rats by the oral or i.v. route (Cumulative per cent of radioactive dose administered)

Sampling Time [h]	Dose (mg/kg bw)				
	2			200	
	M, oral	M , i.v.	F, oral	M, oral	F, oral
	Mean± CV[%]	Mean± CV[%]	Mean± CV[%]	Mean± CV[%]	Mean± CV[%]
Faeces					
24	20.55±9.45	13.31±20.60	6.86±19.85	20.22±12.73	4.90±23.25
48	22.82±4.60	14.41±21.60	7.38±19.62	25.84±7.78	9.64±15.13
72	23.09±4.31	14.64±22.29	7.49±19.61	26.14±8.01	10.32±13.70
Urine					
4	17.25±29.64	26.12±12.06	5.34±26.95	14.89±13.69	9.50±30.43
8	40.99±14.13	44.02±12.36	33.80±48.61	31.33±25.20	28.56±20.08
12	51.67±11.97	NA	NA	NA	NA
24	72.61±2.19	73.80±4.41	86.95±2.91	68.26±3.68	72.15±6.78
48	75.15±2.23	75.75±3.60	89.77±2.36	75.56±3.29	84.15±5.06
72	75.45±2.25	76.24±3.58	90.07±2.31	76.26±2.91	85.95±4.31
Sum	**98.55±1.02**	**90.88±1.94**	**97.56±1.48**	**102.40±0.51**	**96.26±4.70**

Data excerpted from pages 181, 186, 191, 196, and 201 of the study report.

Metabolism

Urine

Over 48 hours, the main compound in urine was parent BYI 02960 which represented approximately 38%, 44% and 36% of the total dose administered in male rats administered 2 mg/kg bw orally, 2 mg/kg bw by i.v. and 200 mg/kg bw orally, respectively. The amount of the parent BYI 02960 in the urine of female rats following oral administration was higher and accounted for approximately 74% of the administered dose in the low (2 mg/kg bw) dose group and approximately 61% in the high (200 mg/kg bw) dose group. Eight metabolites were detected in urine, which included one major metabolite BYI 02960-OH that accounted for approximately 18%, 16% and 13% of the dose in males administered 2 mg/kg bw orally, 2 mg/kg bw by i.v., and 200 mg/kg bw orally, respectively, and approximately 9% and 12% in females administered 2 and 200 mg/kg bw orally, respectively. Two further metabolites were prominent metabolites in the urine of male rats, BYI 02960-hippuric acid accounting for approximately 7%, 5% and 10% for the 2 mg/kg bw oral, 2 mg/kg bw i.v. and 200 mg/kg bw oral dose groups respectively, and BYI 02960-6-CNA accounting for approximately 2%, 3% and 6% in the corresponding dose groups. These two metabolites were also detected in the urine of female rats but at lower amounts of only approximately <1% – 2% of the administered dose. Other identified metabolites were BYI 02960-des-difluoroethyl, BYI 02960-OH-gluA (isomer 1 and 3), BYI 02960-OH-SA and BYI 02960-iso-OH, which accounted for less than 3% of the dose, each (Table 8).

In total over 48 hours post dosing, approximately 75 - 76% of the total dose were detected in urine of male rats for both doses and routes of administration, and approximately 90% and 84% in the urine of female rats administered 2 and 300 mg/kg bw by the oral route, respectively. Identified metabolites in urine represented approximately 71 – 72% of the total administered dose in male rats for both doses and routes of administration, and approximately 88% and 81% in female rats administered 2 and 300 mg/kg bw by the oral route, respectively. In total 19 unknown metabolites were characterised but not identified by their HPLC behaviour. All of them were ≤1% of the administered dose and most of them at trace levels of only 0.1 and 0.2% of the dose in both sexes. The characterised metabolites represented in total approximately 4 -5% of the administered dose in males and 2 - 3% in females (Table 8).

Table 8: Percent of dose of parent compound and metabolites in urine and faeces

Sex		**M**			**F**			**M**			**F**			**M**		
Dose [mg/kg bw] /route of administration		**2 (oral)**			**2 (oral)**			**200 (oral)**			**200 (oral)**			**2 (i.v.)**		
Sample		**U**	**F**	**E**	**U**	**F**	**E**	**U**	**F**	**E**	**U**	**F**	**E**	**U**	**F**	**E**
Time [h]		**0-48**	**0-24**	**sum**	**0-48**	**0-24**	**sum**	**0-48**	**0-24**	**sum**	**0-48**	**0-48**	**sum**	**0-48**	**0-24**	**sum**
% of dose administered		**75.2**	**20.5**	**95.7**	**89.8**	**6.9**	**96.6**	**75.6**	**20.2**	**95.8**	**84.2**	**9.6**	**93.8**	**75.8**	**13.3**	**80.1**
Peak ID	**Compound (report name) BYI 2960-**															
26	**Parent**	**37.6**	**3.3**	**40.9**	**73.8**	**3.9**	**77.7**	**36.1**	**3.5**	**39.6**	**61.1**	**4.4**	**65.5**	**43.9**	**3.4**	**47.3**
7	6-CNA	2.3	0.1	2.4	0.4	---	0.4	6.0	0.2	6.3	1.3	---	1.3	2.7	0.1	2.8
9	hippuric acid	7.4	0.2	7.6	1.1	---	1.1	10.4	0.2	10.5	2.2	0.1	2.2	5.1	<0.1	5.1
`14	OH-gluA (isomer 1)	1.8	---	1.8	0.4	---	0.4	1.6	---	1.6	0.7	---	0.7	1.6	---	1.6
`16	OH-gluA (isomer 3)	2.3	0.1	2.4	0.4	---	0.4	2.3	0.1	2.3	1.0	<0.1	1.1	1.0	---	1.0
18	des-difluoroethyl	2.0	0.2	2.2	2.3	0.1	2.4	1.4	0.4	1.8	2.5	0.2	2.7	1.5	0.2	1.7
24	OH-SA	0.1	0.1	0.2	0.2	0.1	0.3	0.2	0.2	0.5	0.4	0.1	0.5	0.1	0.1	0.2
25	OH	17.8	11.2	28.9	9.0	1.8	10.8	12.7	11.3	24.0	11.8	3.3	15.1	5.7	6.6	22.3
27	iso-OH	<0.1	0.4	0.4	---	<0.1	<0.1	<0.1	0.4	0.4	---	0.1	0.1	0.1	0.4	0.5
Total identified		**71.4**	**15.5**	**86.9**	**87.7**	**6.0**	**93.7**	**70.7**	**16.2**	**86.9**	**81.0**	**8.2**	**89.2**	**71.7**	**10.8**	**82.5**
1	unknown 1	<0.1	0.2	0.2	<0.1	---	<0.1	0.1	0.2	0.3	0.1	---	0.1	0.1	0.2	0.3
2	unknown 2	0.6	0.1	0.7	0.1	---	0.1	1.0	0.1	1.1	0.3	---	0.3	0.6	---	0.6
3	unknown 3	0.2	0.2	0.5	0.1	---	0.1	0.4	0.2	0.5	0.1	---	0.1	0.3	0.1	0.4
4	unknown 4	0.3	---	0.3	0.1	---	0.1	0.4	0.1	0.5	0.3	---	0.3	0.3	---	0.3

5	unknown 5	0.1	0.2	0.3	0.1	---	0.1	0.1	0.1	0.2	0.1	---	0.1	0.1	---	0.1
5a	unknown 6	---	0.2	0.2	---	---	---	---	0.1	0.1	---	---	---	---	---	---
6	unknown 7	0.4	0.1	0.5	0.2	---	0.2	0.1	0.1	0.2	0.1	---	0.1	0.3	---	0.3
8	unknown 8	0.1	<0.1	0.2	0.2	---	0.2	0.5	---	0.5	0.3	---	0.3	0.3	---	0.3
10	unknown 9	0.2	0.2	0.4	<0.1	---	<0.1	0.1	0.2	0.3	0.1	<0.1	0.1	0.1	0.1	0.2
11	unknown 10	0.1	---	0.1	0.1	---	0.1	0.4	---	0.4	0.3	---	0.3	0.2	---	0.2
12	unknown 11	0.7	0.2	0.9	0.2	0.1	0.3	0.6	0.2	0.8	0.4	0.1	0.6	0.4	0.1	0.5
13	unknown 12	0.2	0.1	0.2	0.1	<0.1	0.1	0.1	0.1	0.2	0.2	<0.1	0.2	0.3		0.3
15	unknown 13	0.3	0.1	0.4	0.3	---	0.3	0.3	0.1	0.4	0.2	---	0.2	0.3	<0.1	0.3
17	unknown 14	0.1	0.1	0.2	0.2	---	0.2	0.2	0.1	0.3	0.2	---	0.2	0.2	---	0.2
19	unknown 15	---	---	---	0.1	---	0.1	---	---	---	0.2	<0.1	0.2		---	
20	unknown 16	0.3	0.1	0.5	0.1	---	0.1	0.4	0.2	0.6	0.1	---	0.1	0.4	0.1	0.5
21	unknown 17	---	---	---	---	---	---	---	---	---	---	---	---	0.1	---	0.1
22	unknown 18	---	---	---	---	---	---	<0.1	---	<0.1	---	---	---	0.1	---	0.1
23	unknown 19	0.1	0.1	0.2	0.1	<0.1	0.1	0.1	0.1	0.2	0.1	---	0.1	0.1	0.1	0.3
Total Characterised		**3.8**	**2.0**	**5.8**	**2.0**	**0.1**	**2.1**	**4.8**	**1.9**	**6.7**	**3.2**	**0.3**	**3.4**	**4.1**	**0.7**	**4.7**
Total in urine/faeces extracts		75.2	17.5	92.7	89.8	6.1	95.9	75.6	18.1	93.7	84.2	8.5	92.7	75.8	11.4	87.2
Solids		---	3.0	3.0	---	0.8	0.8	---	2.1	2.1	---	1.1	1.1	---	1.8	1.8
Losses		---	<0.1	<0.1	---	<0.1	<0.1	---	<0.1	<0.1	---	<0.1	<0.1	---	<0.1	<0.1
Samples not analysed		0.3	2.5	2.9	0.3	0.6	0.9	0.7	5.9	6.6	1.8	0.7	2.5	0.5	1.3	1.8
Total		**75.5**	**23.1**	**98.6**	**90.1**	**7.5**	**97.6**	**76.3**	**26.1**	**102.4**	**86.0**	**10.3**	**96.3**	**76.2**	**14.6**	**90.9**

Note: Samples not analysed were faeces 24 – 48 h and 48 – 72 h in low dose males (2 mg/kg bw by the oral and i.v. route), low dose females (2 mg/kg bw by the oral route), high dose males (200 mg/kg be by the oral route), and faeces 48 – 72 h in high dose females (200 mg/kg bw by the oral route).

U = urine
F = faeces
E = excreta

Data excepted from pages 65 to 69 of the study report (Note: values in urine 0-4 h, 4-8 h, 8-12 h, 12-24 h and 24-48 h are not included in Table 8)

Faeces

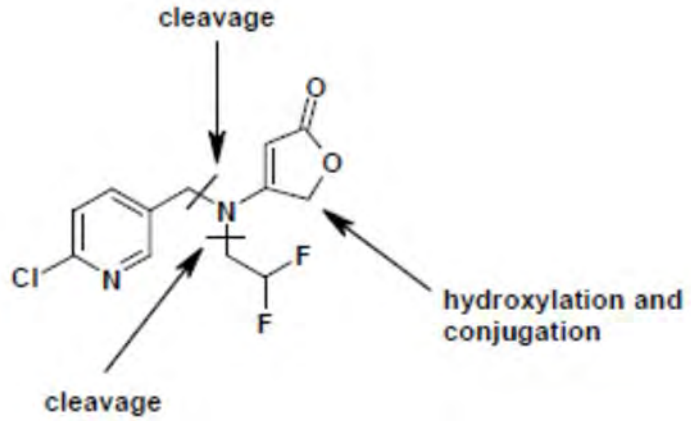
Over 24 hours for all dose groups with the exception of high dose (200 mg/kg bw) females (over 48 hours), parent compound BYI 02960 was the main component of faeces radioactivity in females accounting for approximately 4% of the total administered dose for both oral dose groups (2 and 200 mg/kg bw). In contrast, metabolite BYI 02960-OH was the main component in males faeces, accounting for approximately 11% for both oral dose groups (2 and 200 mg/kg bw0 and the i.v. dose group (2 mg/kg bw). BYI 02960-OH was present in female faeces at approximately 2 - 3% in low (2 mg/kg bw) and high (200 mg/kg bw) oral dose groups, while parent compound BYI 02960 was present in male faeces at approximately 3 – 4% in low and high oral dose groups and the low i.v. dose group. All other identified metabolites, except BYI 02960-OH-gluA, isomer 1, were also detected in faeces but at trace amounts of ≤0.4% of the dose (Table 8).

In total over 24 hours, approximately 13 – 21% of the total administered dose was detected in faeces of male rats of which approximately 11 – 16% were identified and approximately 1 – 2% characterised but not identified. In females over 24 hours for the low dose (2 mg/kg bw) and 38 hours for the high dose 9200 mg/kg bw), approximately 7 – 10% of the total administered dose was detected in faeces of which approximately 6 – 8% was identified and approximately 0.1 – 0.3% characterised but not identified. In total 16 unknown metabolites were characterised but not identified by their HPLC behaviour. All of them were trace metabolites at ≤0.2% of the administered dose (Table 8).

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

The principal metabolic reactions of [pyridinylmethyl-14C]BYI 02960 in rats were:
- hydroxylation followed by conjugation with glucuronic acid to or with sulphate;
- cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl; and
- cleavage of the molecule at the pyridinylmethyl bridge forming BYI 02960-6-CNA, which was further conjugated with glycine to BYI 02960-hippuric acid.

The following figure shows schematically the positions in the molecule, which are involved in the metabolic reactions:



The proposed biotransformation pathway of [pyridinylmethyl-14C] BYI02960 is presented in Figure 3.

III. CONCLUSION

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

- The test compound was completely absorbed in all tests as demonstrated by the high bioavailability after oral administration and by the high amounts of radioactivity detected in urine and the body without GIT at sacrifice.
- The distribution of the radioactivity within the body was rapid and the maximum plasma level (C_{max}) was reached within one hour after administration in the low dose tests and within approximately 2 to 4 hours in the high dose tests. From the maximum, the radioactivity level declined slowly down to approximately 50% of C_{max} after 4 – 8 hours in the low dose tests and after 8 – 24 hours in the high dose tests. The plasma concentrations showed a further decline down to low values around the LOQ in the low dose tests and to approx. 0.5% of C_{max} in the high dose tests.
- Excretion of BYI 02960 related radioactivity was fast and occurred mainly by the renal route. Female rats exhibited slightly higher renal excretion rates of approximately 86% and 90% of the oral administered dose than males with approximately 76% of the dose.
- The major part of the dose (>84%) was excreted within 24 hours after treatment, though excretion was ongoing in the time range between 24 and 72 hours.
- At the time of sacrifice, 72 hours after administration, the residues were low and only trace amounts of approx. <0.1 – 0.3% of the dose administered was detected in the body and in the GIT. Most of the residues of the low dose tests were lower than 0.01 mg/kg sample. The highest concentrations, but also at a low level of approximately ≤0.018 mg/kg sample were detected in blood cells, in the GIT of male rats and in the eyes of female rats. The residues of the low and high dose tests were nearly proportional to the dose.

- Parent compound, three major and five minor metabolites were identified in all samples. Identification rates were high and amounted between approximately 83% and 94% of the dose administered.
- In total a further 19 unknown metabolites were characterised by their chromatographic behaviour but not identified. All of them were detected at trace amounts of approx. <0.1% to 0.9% of the total dose administered.
- The metabolic transformation of BYI 02960 was principally oxidative and took place at least at 3 different structural positions of the test compound.
- The results of the present study were in good accordance with those obtained in the other rat studies performed with different radiolabels: [Ethyl-1-^{14}C]BYI 02960 (Weber, 2011a; Study No. M1824567-3, Koester, 2012a; Study No. M1824575-2) and [Furanone-4-^{14}C]BYI 02960 (Weber, 2011b; Study No.M1824560-6, Koester, 2012b; Study No. M1824574-1, Koester, 2011b, Study No. M1821760-5).

Based on the results obtained the absorption, distribution, excretion, and metabolism of[pyridinylmethyl-^{14}C]BYI 02960 in rats are assumed as adequately understood.

Figure 3. Proposed metabolic pathway of [Pyridinylmethyl-^{14}C]BYI 0296 in the male rats

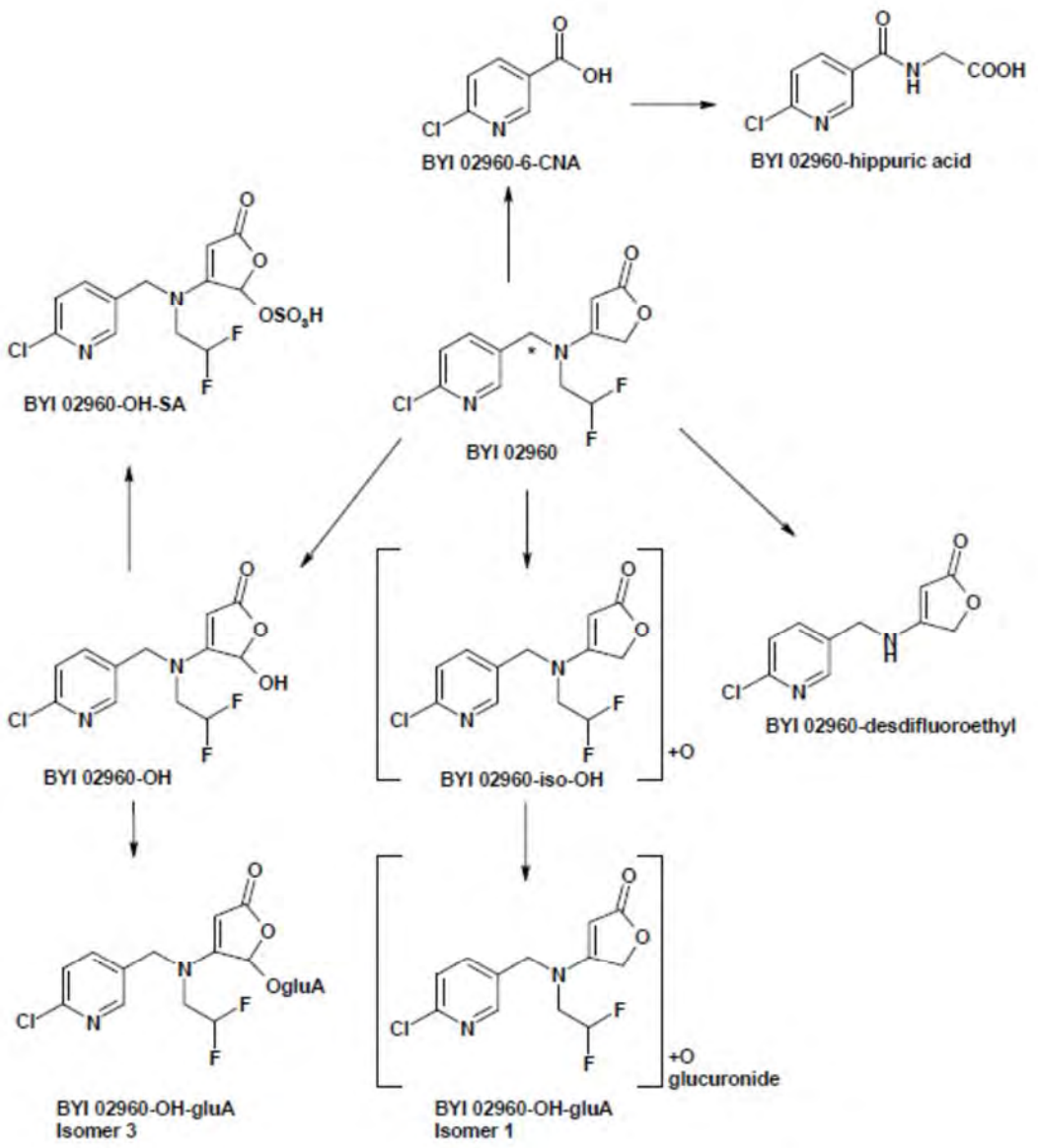


Figure extracted from page 156 of the study report.

Primary review: Australia
Secondary review: US EPA

IIA 5.1.1/06 Whole body distribution study in rats: [pyridinylmethyl-^{14}C]BYI 02960

Report: Koester, J (2011a) Quantitative whole body autoradiography of [pyridinylmethyl-^{14}C]BYI 02960 in male and female rats: distribution of total radioactivity and elimination from blood, organs and tissues after single oral administration including determination of radioactivity in the excreta and exhaled $^{14}CO_2$. Bayer CropScience AG, Development - Environmental Safety, Metabolism/ADME and Environmental Fate, D-40789 Monheim am Rhein, Germany. Study No. M1821837-0. May. MRID 48844142. Unpublished.

Guidelines: US EPA 870.7485 (1998)
OECD TG 417 (2010)
JMAFF 12 Nousan 8147 (2001 amendment)
PMRA Dir2005-01: Section 11 (2005)
EC No 1107/2009 (2009)

Deviations: None

GLP: Yes. Signed and dated GLP, Quality Assurance and Data Confidentiality statements were presented in the report.

Executive summary:

In a quantitative autoradiography kinetic study, 8 male and 8 female Wistar rats were administered by oral gavage a single dose of 5 mg/kg bw [pyridinylmethyl-^{14}C]BYI02960 in 0.5% aqueous Tragacanth®. The test compound was radiolabelled with ^{14}C in the pyridinylmethyl bridge of the molecule. A single animal of each sex was sacrificed by carbon dioxide asphyxiation at selected time points from 1 hour to 168 hours post dosing, and urine and faeces samples were also taken up to sacrifice along with carbon dioxide for selected time periods and the amounts of radioactivity in the samples determined. The distribution of total radioactivity to and elimination from blood, organs and tissues were also analysed qualitatively and quantitatively in sacrificed animals by whole-body autoradioluminography. The control animals (a single male and female) were sacrificed at 4 hours, for detection of possible chemo-graphic effects, and demonstrated no such effects.

BYI 02960 was readily absorbed from the gastrointestinal tract and distributed throughout the body immediately after administration. The excretion of radioactivity via urine and faeces was almost completed after 2 days with renal excretion as the predominant route. For male rats, by 168 hours post dosing approximately 81% in males and 92% in females of the administered dose were recovered in urine and approximately 22% in males and 8% in females in the faeces. Less than 0.1% of the administered dose was exhaled as $^{14}CO_2$ or other volatiles during a sampling period of 48 hours. This demonstrated the stability of the pyridinylmethyl-^{14}C label with regard to formation of volatile products.

In male and female rats, maximum TRR-values (= CEQmax: expressed as µg a.s. equiv. /g) were reached for nearly all organs and tissues at one hour after administration (= tmax). At this time, the values for liver and kidney were significantly higher than in blood, suggesting a preferred clearance from blood and distribution mainly to those organs that are mainly responsible for metabolism (liver) and excretion (kidney), along with several glands. Very low levels were found in the brain, spinal chord and in renal fat. From peak values, a fast decline of radioactivity concentrations was observed for all organs and tissues in males and females during the whole testing period.

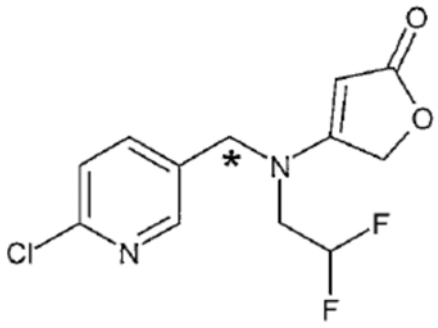
In both sexes, concentrations fell for most organs and tissues below 5% of the maximum after one day and below the limit of quantification after seven days post administration. After seven days, only minor to very low concentrations of radioactivity were found in a few organs and tissues. Overall, male and female rats exhibited a very similar absorption, distribution and excretion behaviour. From the results of this study, any accumulation or significant retention of [pyridinylmethyl-14C] BYI 02960 in male and female rats can be excluded.

This study is fully reliable (acceptable/guideline) and satisfies the study requirements of a toxicokinetics study, albeit through use of an atypical method of radioactivity detection and quantification (U.S. EPA 870.7485; OECD Section 4 Part 417; JMAFF 12 Nousan 8147; PMRA Dir2005-01: Section 11; EC No 1107/2009).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI02960
   Batch #: BYI 02960-PU-02
   Purity: 99.4%
   Description: Beige powder
   CAS #: 951659-40-8
   Stability of test compound: Stated to be stable for two years if stored at 5±5ºC (assay date 27 Oct 2008).
2. Radiolabeled test material: [pyridinylmethyl-^{14}C]BYI02960
   Lot/Batch #: BECH 2123
   Chemical structure /positions of radiolabel:



* Denotes position of radiolabel

Radio-chemical purity:	>99% (HPLC)
Specific activity:	[pyridinylmethyl-^{14}C] 118.08 Ci/mg
Description:	Solid, dried in vacuum
Stability of test compound:	The positions of the radiolabels are considered to be stable.

3. Vehicle and/or positive control: 0.5% aqueous Tragacanth®
4. Test animals

Species:	Rat
Strain:	Wistar Hsd/Cpb: WU
Age at dosing:	6 weeks (males), 9 weeks (females)
Weight at dosing:	202 to 206 (males), 187 to 210 g (females)
Source:	Harlan Netherlands BV, Kreuzelweg 53, NL-5960 AD Horst, Netherlands.
Acclimation period:	Seven days in Makrolon® metabolism cages prior to dosing.
Diet:	Rat/mice maintenance long life diet No. 3883.0.15, (Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland), *ad libitum* while in metabolism cages, except for approx. 16 hours prior to, and for 6 hours after dosing.
Water:	Tap water, *ad libitum*
Housing:	Individually housed in Makrolon® metabolism cages with wood floor shavings. Cages permitted an 'almost' quantitative separate urine and faeces collection.

5. Environmental conditions

Temperature:	19 to 24°C
Humidity:	49 to 61%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

6. Preparation of the dosing solutions

Rats were dosed with [pyridinylmethyl-^{14}C] BYI02960. The [pyridinylmethyl-^{14}C] BYI02960 radiolabel was diluted with technical BYI02960. The dose vehicle 0.5% aqueous Tragacanth® was added using an ultrasonic bath and stored at 5°C until use and kept homogenous by magnetic stirring bars and glass balls. Dose route, dose level, volume, and radiolabel data are presented in Table 1. The test compound was administered by a single oral gavage dose.

Table 1. Dose Levels, Volumes, and Radiochemical Data

Test No.	Administration suspension							
	Total prepared		Administered per rat					
	Amount (mg)	Volume (mL)	Volume/ Amount	Target dose (mg/kg bw)	Radioactivity (dpm)	Amount (mg)	Actual dose (mg/kg bw)	Mean rat weight (g)
1 (M)	12	24	2.0 mL /1.0 mg	5	261, 326, 058	1.0	4.89	204
2 (F)	12	24	2.0 mL /1.0 mg	5	270, 664, 944	1.03	5.27	196

Note: The study also included a control male and female rat which did not receive the radiolabelled test material

Data excerpted from page 20 of the study report.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

   21 Jan 2009 to 30-May-2011

2. Study design

   The basis for the dosing regimen of 5 mg/kg bw was not reported. Animals were assigned to test groups according to Table 2.

Table 2. Study design

Group	No. of animals	Achieved oral dose (mg/kg bw)	Sampling intervals or timepoint (hours)			
			Urine	Faeces	Expired Air*	Whole body including whole blood
1	8 males	4.89	0 – 1, 1 – 4, 4 – 8, 8 – 24, 24 – 48, 48 – 72, 72 – 96, 96 – 120, 120 – 144, 144 – 168	0 – 24, 24 – 48, 48 -72, 72 – 96, 96 – 120, 120 – 144, 144 – 168	0 – 24, 24 – 48	1, 4, 8, 24, 48, 72, 120, 168 (at time of sacrifice)
2	8 Females	5.27				

*Carbon dioxide from expired air was collected from 4 animals in Group 1 and Group 2

Note: A single animal from Group 1 and Group 2 was sacrificed at each of the times given for whole body sampling. Control animals (a single male and female not shown in the table above) were sacrificed at 4 h, and no urine, faeces or expired air samples were obtained from these animals. The control animals were used for detection of possible chemo-graphic effects.

3. Tissue Distribution at sacrifice

At the designated sacrifice time (see Table 2 above) animals in each group including control animals were sacrificed by carbon dioxide asphyxiation . Sacrificed animals were immediately deep frozen in a solid carbon dioxide/ dichloromethane mixture at approximately -70 °C and embedded in aqueous carboxymethylcellulose (CMC) gel (7-8%). Sagittal sections (from cranial to caudal) were cut with a thickness of 50 µm at ca. -25 °C using a cryomicrotome, slightly sprayed with a liquid plaster to stabilise the whole section and dehydrated for at least 24 hours. Four to five sections showing the relevant organs and tissues of the respective body planes (e.g. eye-kidney) were prepared from each animal. The distribution of total radioactivity (i.e. without differentiation of unchanged test compound and radiolabelled biotransformation products) in rat organs and tissues was then determined qualitatively at the various time-points by whole-body autoradioluminography employing the Fuji BAS 5000® phosphorimaging system.

4. Quantitation of Radioactive Residues

Radioactivity in plasma or blood cells could not be determined separately due to the autoradiographic sampling technique, and therefore only whole blood concentrations were determined. The measurement of the radioactivity in liquid samples (which included funnel rinses from the Makrolon® metabolism cage for urine samples) was carried out by LSC. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

Whole-body radiography was used to quantify radioactivity in a given tissue or organ. This method allows visualisation and quantitative evaluation of selective enrichments of radioactivity, particularly in certain parts of organs or tissues of the animal. The quantification of equivalent concentrations in the organ and tissues was performed using ^{14}C-spiked blood standards for calibration, with the radioactivity concentration of this standard being determined by LSC.

II. RESULTS AND DISCUSSION

A. STORAGE STABILITY

BYI02960 stated to be stable for two years if stored at 5±5ºC. No other storage stability data were provided.

B. MAIN STUDY

Clinical Observation

Clinical signs were not discussed in the study report.

Absorption and Ddistribution

Gastrointestinal absorption of the test substance was rapid with extensive distribution. In both sexes, maximum equivalent concentrations (tmax) were reached in nearly all organs and tissues at the first sampling time of 1 hour post-dosing. At tmax, the organ/blood radioactivity concentration ratio values for many organs and tissues in both sexes were in a range of approximately 0.8 – 1.2. In both sexes, distribution was seen to be greatest towards those organs or tissues responsible for metabolism (liver, tissue/blood concentration value 1.78 in males, 1.79 females), excretion (kidney, 1.71 males, 1.65 females) and secretion (e.g. adrenal, 1.77 males, 1.75 females, thyroid, 1.34 in both sexes, Harderian, 1.41 males, 1.21 females) and salivary glands (1.3 in both sexes). The lowest values were measured for perirenal fat (0.14 males, 0.16 females), the spinal cord (0.42 males, 0.32 females), and brain (0.38 males, 0.31 females).

The equivalent concentrations in blood, organs and tissues in both sexes declined rapidly with an approximately first order kinetics in the time range between 1 and 48 hours. At 24 hours post dosing, the concentrations in blood and also in most other tissues were below 5% of the maximum concentration in both sexes, except nasal mucosa and vitreal body with approximately 15% of the CEQmax in males and 7-11% in females. At 48 hours, CEQmax values in both sexes had declined to below 1% in blood and most tissues, except vitreal body and nasal mucosa which had CEQmax values of approximately 5-8% in males and 3-4% in females.

At 168 hour post dosing in both sexes, the equivalent concentrations in nearly all organs and tissues had declined below the limit of quantification. Very low residual concentrations of radioactivity (<0.1mg/kg sample) were still present in the eye (vitreal body, females only), blood, renal medulla, liver (females only), and lung. For all these organs, the concentration was below the value for blood. The tissue/blood concentration ratio was exceptionally high for the nasal mucosa (factor 4.1 males, 5.53 females), but this corresponded to a very low absolute level of 0.037 mg/kg sample (Table 3).

Since no blackening of typical animal structures could be observed in control animals, chemo-graphic effects were excluded for the imaging data obtained for the sections of the animals treated with the radiolabelled test compound.

Excretion

Over the study period, in both sexes the major part of the total dose administered was excreted in the urine (approximately 81% in males, 92% in females) and a minor part in faeces (approximately 22% in males, 8% in females), and a negligible amount of <0.1% exhaled in both sexes over a 48 hour period from 48 hours post dosing. This demonstrated that the pyridinylmethyl radio label of the molecule was completely stable with regard to formation of carbon dioxide.

Thus, it can be seen that females exhibited a slightly higher renal excretion rates than males. Furthermore, excretion was rapid, with approximately 93% in males and 97% in females of the total administered radioactivity excreted by 24 hours post dosing, and the excretion nearly complete in both sexes by 48 hours (Table 4).

Table 3. Individual equivalent concentrations of radioactivity in blood, organs and tissues (equivalent concentration CEQ [µg a.s. equiv. /g])

Organ/Tissue	Male - Animal No.								Female - Animal No.							
	785	**786**	**787**	**788**	**789**	**790**	**791**	**792**	**794**	**795**	**796**	**797**	**798**	**799**	**800**	**801**
	Time of sacrifice (hours post dosing)															
	1	**4**	**8**	**24**	**48**	**72**	**120**	**168**	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Blood	**3.171**	2.376	1.692	0.122	0.026	0.020	0.013	0.009	**4.155**	3.211	2.005	0.090	0.016	0.012	0.010	0.007
Liver	**5.645**	4.326	3.332	0.246	0.027	0.014	0.008	0.005	**7.451**	5.878	3.778	0.171	0.016	0.008	0.006	<LOQ
Renal cortex	**4.441**	3.267	2.565	0.146	0.016	0.007	0.005	<LOQ	**5.460**	4.414	2.834	0.124	0.009	0.005	0.005	<LOQ
Renal medulla	**6.398**	4.538	3.680	0.248	0.021	0.013	0.008	0.006	**8.212**	7.083	4.877	0.222	0.013	0.009	0.007	0.008
Kidney total	**5.420**	3.903	3.122	0.197	0.019	0.010	0.006		**6.836**	5.748	3.856	0.173	0.011	0.007	0.006	
Brown fat	**3.288**	2.479	2.023	0.098	0.007	---	---	---	**3.229**	3.174	1.811	0.095	<LOQ	---	---	---
Perirenal fat	**0.446**	0.351	0.219	0.013	<LOQ	<LOQ	<LOQ	<LOQ	0.413	**0.502**	0.315	0.019	<LOQ	<LOQ	<LOQ	<LOQ
Skeleton muscle	**3.246**	2.283	1.608	0.096	0.005	<LOQ	<LOQ	<LOQ	**3.746**	3.140	1.926	0.075	<LOQ	<LOQ	<LOQ	<LOQ
Myocardium	**4.426**	3.155	2.250	0.142	0.007	<LOQ	<LOQ	<LOQ	**5.198**	4.474	2.701	0.105	0.006	<LOQ	<LOQ	<LOQ
Lung	**2.450**	2.168	1.373	0.082	0.015	0.013	0.005	0.005	**3.230**	1.142	1.311	0.054	0.008	0.007	0.005	0.005
Spleen	**2.873**	2.093	1.557	0.096	0.010	<LOQ	<LOQ	<LOQ	**3.742**	2.975	1.932	0.077	<LOQ	<LOQ	<LOQ	<LOQ
Pancreas	**4.089**	2.882	2.170	0.129	0.005	<LOQ	<LOQ	<LOQ	**5.245**	3.961	2.626	0.100	<LOQ	---	<LOQ	<LOQ
Bone marrow	**2.770**	1.825	1.391	0.083	---	---	---	---	**2.843**	2.344	1.476	0.062	---	---	---	---
Gonads	**2.251**	2.198	1.466	0.092	<LOQ	<LOQ	<LOQ	<LOQ	**3.688**	3.101	1.915	0.077	0.006	<LOQ	<LOQ	---
Uterus	NA								**3.950**	3.262	2.052	0.081	<LOQ	<LOQ	<LOQ	<LOQ
Brain	**1.214**	0.852	0.619	0.036	<LOQ	<LOQ	<LOQ	<LOQ	**1.279**	1.052	0.626	0.026	<LOQ	<LOQ	<LOQ	<LOQ
Spinal cord	**1.334**	1.004	0.777	0.042	<LOQ	---	---	---	**1.348**	1.074	0.692	0.029	<LOQ	---	---	---
Pituitary gland	**3.539**	2.435	1.889	0.105	---	---	---	---	**4.306**	3.793	2.293	0.082	---	---	---	---
Pineal body	**3.033**	1.488	1.680	0.089	0.005	<LOQ	---	---	**3.427**	2.797	1.914	0.075	---	---	---	---
Adrenal gland	**5.626**	4.429	3.338	0.213	0.017	0.007	<LOQ	<LOQ	**7.289**	5.669	4.029	0.201	0.017	0.008	<LOQ	<LOQ
Thymus	**3.173**	2.328	1.646	0.096	<LOQ	<LOQ	<LOQ	<LOQ	**4.090**	3.180	1.971	0.075	<LOQ	<LOQ	<LOQ	<LOQ
Thyroid gland	**4.252**	3.284	2.219	0.143	0.012	<LOQ	---	---	**5.548**	4.284	2.809	0.102	0.006	---	---	---
Salivary gland	**4.123**	3.097	2.195	0.131	0.008	<LOQ	<LOQ	<LOQ	**5.411**	4.177	2.642	0.102	0.006	<LOQ	<LOQ	<LOQ
Nasal mucosa	1.409	**1.477**	1.210	0.250	0.120	0.077	0.043	0.037	1.585	**2.381**	1.720	0.164	0.102	0.068	0.047	0.037
Vitreal body	**1.575**	1.370	1.459	0.273	0.073	0.036	0.011	<LOQ	**2.303**	2.065	1.376	0.248	0.062	0.024	0.009	0.008
Harderian gland	**4.469**	3.083	2.415	0.151	0.014	---	---	---	**5.008**	4.048	2.483	0.107	0.019	<LOQ	---	---

Note: Maximum CEQ - values (= CEQmax) are shown in bold (1 hour was found to be CEQ_{max} for all tissues and blood, with the exception of the nasal mucosa in males where it was 4 hours, and the perirenal fat and nasal mucosa of females, where it was 4 hours. – denotes that organ or tissue was visible in the rat sections but not discernible in the radioluminograms.

Data excerpted from pages 34 and 38 of the study report.

Table 4. Cumulative excretion of radioactivity in urine, faeces and exhaled air of rats

	Percent of radioactive dose administered															
	Male - Animal No.								**Female - Animal No.**							
	785	**786**	**787**	**788**	**789**	**790**	**791**	**792**	**794**	**795**	**796**	**797**	**798**	**799**	**800**	**801**
	Time of sacrifice (hours)															
	1	**4**	**8**	**24**	**48**	**72**	**120**	**168**	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
	Exhaled air															
24					0.06	0.06	0.07	0.06					0.02	0.02	0.02	0.02
48					0.07	0.08	0.09	0.09					0.02	0.03	0.02	0.03
	Urine															
1	2.38								7.19							
4		22.39	12.55	14.62	2.71	2.16	2.03	11.86		9.79	9.69	12.35	11.53	3.55	25.51	4.35
8			42.07	45.19	23.14	30.59	44.03	29.61			32.74	33.40	27.68	49.84	26.53	4.41
24				78.07	68.52	79.11	76.13	75.12				86.47	84.60	86.82	90.42	78.34
48					72.34	84.54	79.85	79.67					89.90	88.76	92.95	88.13
72						85.12	80.35	80.05						88.94	93.16	89.93
96							80.53	80.19							93.23	91.15
120							80.70	80.29							93.28	91.61
144								80.38								91.95
168								80.50								92.46
	Faeces															
24	*****	*****	*****	14.93	23.63	17.37	20.26	17.49	*	*	*	10.62	8.61	8.75	6.70	4.54
48					26.75	20.34	22.49	21.59					10.58	9.75	7.82	6.98
72						20.54	22.74	21.92						9.78	7.89	7.36
96							22.82	21.98							7.92	7.47
120							22.85	22.04							7.93	7.49
144								22.05								7.52
168								22.12								7.53
Sum total	**2.38**	**22.39**	**42.07**	**92.99**	**99.17**	**105.74**	**103.64**	**102.71**	**7.19**	**9.79**	**32.74**	**97.09**	**100.50**	**98.76**	**101.23**	**100.03**

* – denotes faeces not collected.

Data excerpted from page 37 and page 41 of the study report.

III. CONCLUSION

BYI 02960, labelled with ^{14}C in the pyridinylmethyl-position of the molecule, was readily absorbed from the gastrointestinal tract of male and female Wistar rats after single oral administration. Test compound related radioactivity was distributed throughout the bodies immediately after administration however with a clear preference to those organs or tissues that are responsible for metabolism, excretion and secretion.

Peak concentrations of radioactivity for nearly all organs and tissues were reached at the first sampling point of one hour after dosing. From the peak values, the concentrations declined rapidly and fell for most organs and tissues below 5% of the maximum after one day and below the limit of quantification after seven days post administration.

The radioactivity was quickly excreted, mainly by the renal route with a minor part in the faeces. A negligible amount (<0.1%) was exhaled in both sexes. Over the 168 hour study period, a slightly higher proportion of renal excretion was seen in females (approximately 92%) compared to males (approximately 81%. Greater than >90% of the administered dose was excreted within 24 hours in both sexes, and excretion was nearly complete in both sexes by 48 hours (i.e. >99%).

Overall, males and females exhibited a very similar absorption, distribution and excretion profiles, and the results of this study do not provide evidence for accumulation or significant retention of [pyridinylmethyl-14C] BYI 02960 in male and female rats.

Primary review: Australia
Secondary review: US EPA

IIA 5.1.1/03 ADME Study in rats: [furanone-4-^{14}C]BYI 02960

Report: Weber, E (2011b) [Furanone-4-^{14}C]BYI02960 – Absorption, Distribution, Excretion, and Metabolism in the Rat. Bayer CropScience AG Development - Environmental Safety Metabolism/ADME and Environmental Fate D-40789 Monheim am Rhein Germany. Study # M1824560-6. MRID 48844143. Unpublished.

Guidelines: U.S. EPA 870.7485 (1998)
OECD Section 4 Prt (417; 2010)
JMAFF 12 Nousan 8147 (2000)
Deviations: None

GLP: Yes. Signed and dated GLP, Quality Assurance and Data Confidentiality statements were presented in the report.

Executive summary:

In a toxicokinetic and metabolism study (MRID 48844143), a group of 4 male and 4 female Wistar rats were administered by oral gavage a single dose of 2 mg/kg bw [furanone-4-^{14}C]BYI 02960 in 0.5% aqueous Tragacanth® and sacrificed by exsanguination under anaesthesia at 168 hours post dosing. The test compound was radiolabelled with ^{14}C in the C4-position of the furanone ring of the molecule. The metabolism and radioactivity which included the test compound and metabolites was determined in urine and faecal samples collected for the time range between dosing and sacrifice. Additionally, radioactivity in plasma micro samples was determined over the study period (23 time points). Total radioactivity was determined in excreta and in organs and tissues collected at sacrifice. Samples were analysed, and parent compound and metabolites identified, by radiochromatographic (HPLC, TLC) and spectroscopic (LCMS) methods.

Mean total radioactivity recovery was nearly quantitative since approximately 96% and 102% of the administered dose were found in the excreta and bodies of males and females respectively at sacrifice.

[Furanone-4-14C]BYI 02960 was nearly completely absorbed since >79% and >91% of the total dose administered was detected in the urines and the bodies without GIT at sacrifice of male and female rats respectively. The absorption started immediately after dosing as could be seen from the quick increase of radioactivity in plasma micro samples, with the peak plasma concentration (C_{max}) was reached approximately 1.5 hours after administration in both sexes.

The distribution of the radioactivity within the body was fast. From the maximum plasma level (C_{max}), the radioactivity level declined slowly down to approximately 50% of C_{max} after 8 hours in both sexes and <1% of C_{max} 72h after dosing.

Excretion was rapid and mainly by the renal route. Approximately 79% of the total dose administered was found the urine of male rats and approximately 91% in the urine of female rats.

The major part of the dose was excreted within 24 hours after treatment, for male and female rats. At the time of sacrifice, 168 hours after administration, a small proportion of approximately 0.5% of the dose administered for males and approximately 0.2% for females was still detected in the body without GIT.

The parent compound was moderately metabolised to approximately 20 metabolites in total, consisting of one major (BYI 02960-OH) and six minor metabolites of an isolated and purified compound. Identification rates were >84% of the total dose administered in male rats and >95% in females. Another approximate 8% of the dose in males and 4% in females were characterised by their chromatographic behaviour but not identified. All metabolites accounting for >1% of the dose administered were identified except a polar metabolite fraction accounting for 5.30% in males and 2.45% in females. It is considered that this polar fraction is most likely represented by biomolecules. Parent compound represented the predominant part of the radioactivity in urine but in faeces samples the metabolite BYI 02960-OH was more prominent.

Overall, male rats showed a higher rate of metabolism with only approximately 55% of unchanged parent compound found in excreta whereas 76% of unchanged BYI 02960 was found in the excreta of female rats, though the metabolic patterns were very similar in males and females.

The principal metabolic reactions of [furanone-4-^{14}C]BYI 02960 in rats were: (1) hydroxylation followed by conjugation with glucuronic acid or with sulphate; (2) cleavage of the difluoroethyl group forming BYI 02960-desdifluoroethyl; (3) cleavage of the molecule at the pyridinylmethyl bridge forming BYI 02960-difluoroethyl-amino-furanone; and (4) cleavage of molecule at the nitrogen-carbon bond next to the furanone moiety followed by further conversion to C1 and C2 compounds of the natural pool including complete degradation to carbon dioxide.

Male rats exhibited a higher proportion of the polar metabolite fraction and higher residues in the body at the time of sacrifice, and also exhibited a higher proportion of radioactivity in exhaled air in a study with [pyridinylmethyl-^{14}C]BYI 02960 (Koester 2011a). All these observations taken together suggest that the furanone-4-^{14}C radiolabel is not completely stable and that a small part of the dose obviously underwent extensive biotransformation to C1- and C2-fragments that resulted in an incorporation of radioactivity into biomolecules, in particular in male rats.

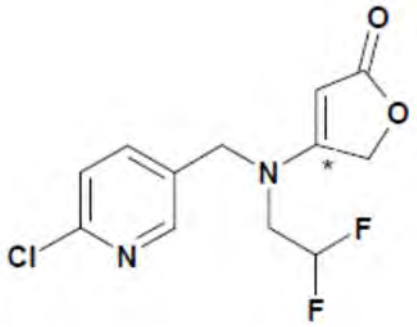
The results of these metabolism investigations are in good accordance with those obtained from the corresponding organ metabolism rat study with [furanone-4-^{14}C]BYI 02960 (Koester, 2012b).

This study is fully reliable (acceptable/guideline) and satisfies the study requirements of a metabolism study (U.S. EPA 870.7485; OECD Section 4 Part 417 and Annex to Directive 87/302/EEC, Part B, Toxicokinetic; JMAFF 12 Nousan 8147).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   Lot/Batch #: KATH 6279
   Purity: >99% (HPLC)
   Description: Solid, dried in vacuo
   CAS #: 951659-40-8
   Stability of test compound: Not reported.
   Chemical structure /position of radiolabel(*):



   Radio-chemical purity: [furanone-4-^{14}C]BYI 02960 >99% (HPLC) >99% (TLC)
   Specific activity: [furanone-4-^{14}C] 106.46 μCi/mg.
   Stability of test compound: The positions of the radiolabels are considered to be stable.
3. Vehicle and/or positive control: 0.5% aqueous Tragacanth®.
4. Test animals
   Species: Rat
   Strain: Wistar Hsd/Cpb: WU
   Age at dosing: 6 weeks (male), 8-9 weeks (female)
   Weight at dosing: 202–212 g (male), 198-203 g (female)
   Source: Harlan Netherlands BV, Kreuzelweg 53, NL-5960 AD Horst, Netherlands.
   Acclimation period: Seven days in Makrolon® metabolism cages prior to dosing.
   Diet: Rat/mice maintenance long life diet No. 3883.0.15 (Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland), *ad libitum*, except for 16 hours prior to dosing and up to six hours post-dosing.
   Water: Tap water, *ad libitum*.
   Housing: Individually in Makrolon® metabolism cages with wood floor shavings. Cages permitted an 'almost' quantitative separate urine and faeces collection.
5. Environmental conditions

Temperature:	22–24°C
Humidity:	45–67%
Air changes:	10–15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

6. Preparation of the dosing solutions

Rats were dosed with [furanone-4-^{14}C]BYI 02960. The [furanone-4-^{14}C]BYI 02960 radiolabel was diluted with technical BYI02960. The dose vehicle 0.5% aqueous Tragacanth® was added using an ultrasonic bath and stored at 5ºC until use and kept homogenous by magnetic stirring bars and glass balls. Dose route, dose level, volume, and radiolabel data are presented in Table 1. The test compound was administered by a single oral gavage dose.

Table 1. Dose Levels, Volumes, and Radiochemical Data

Test No.	**Administration suspension**							
	Total prepared		**Administered per rat**					
	Amount (mg)	**Volume (mL)**	**Volume/ Amount**	**Target dose (mg/kg bw)**	**Radioactivity (dpm)**	**Amount (mg)**	**Actual dose (mg/kg bw)**	**Mean rat weight (g)**
1 (M)	2.4	12	2.0 mL /0.4 mg	2	95, 350, 450	0.40	1.96	206
2(F)	2.4	12	2.0 mL /0.4 mg	2	96, 477, 175	0.41	2.04	200

Data excerpted from pages 19 of the report.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

05 Nov 2011 to 20 June 2011

2. Study design

The dose level was based on the specific radioactivity, stability of the dosing suspensions, and the fact that 2 to 5 mg/kg bw doses used as the low dose levels for ADME studies in rats by Weber (2011a, Study no's: M1824567-3; M1824560-6), Klempner (2011; Study no M1824559-4), and the whole body radiography studies of Koester (2011b, M1821760-5) and Koester (2011a, Study no. M1821837-0) had not produced any observable toxicity. The dose level of 2 mg/kg bw was also stated to be consistent with specifications in OECD, US EPA and Japanese MAFF test guidelines on toxicokinetic studies. Animals were assigned to test groups according to Table 2.

Table 2. Study design

Group	No. of animals	Achieved oral dose (mg/kg bw)	Sampling intervals or timepoint (hours)		
			Urine	Faeces	Plasma microsample
1	4 males	1.96	0 – 4, 4 – 8, 8 – 12, 12 – 24, 24 – 48, 48 – 72, 72 – 96, 96 – 120, 120 – 144, 144 – 168	0 – 24, 24 – 48, 48 –72, 72 – 96, 96 – 120, 120 – 144, 144 – 168	10 min, 20 min, 40 min, 1h, 1.5, 2, 3, 4, 6, 8, 24, 28, 32, 48, 52, 56, 72, 96, 120, 144, 152, 168
2	4 females	2.04			

Note: Time of sacrifice was 168 hours post-dosing for both dose groups.

3. Plasma Kinetics Following Dosing

In-life blood samples from individual animals were obtained by pressing a heparin coated capillary into a small cut in the tail vein which was subsequently closed by tape. Blood samples were separated afterwards into plasma and blood cells by centrifugation. At 72 hours post-dosing animals in each group were sacrificed by transection of the cervical vessels and exsanguination under Pentobarbital-Na anaesthesia. Oozed out blood samples from individual animals was collected in heparinised test tubes and separated afterwards into plasma and blood cells by centrifugation. After weighing, aliquots from the whole plasma sample were taken for determination of total radioactivity by liquid scintillation counting (LSC).

4. Tissue Distribution at Sacrifice

Tissues and carcass were analyzed to determine ^{14}C tissue distribution by combustion/LSC. The following tissues were collected at sacrifice from test animals:

Blood (plasma & RBC)	Liver	Femur
Kidney	Leg muscle	Heart
Lung	Testes (Groups 1)	Ovaries (Group 2)
Uterus (Group 2)	Bone femur	Brain
Spleen	Adrenals	Thyroid
G.I.T & contents	Skin	Harderian gland
Eyes	Perirenal fat	Carcass

5. Quantitation of Radioactive Residues

Radioactive residues of all individual animals were measured in plasma, urine (including cage rinse i.e. funnel rinse from the Makrolon® metabolism cage) and faeces samples, and

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

6. Calculation of toxicokinetic parameters

Standard toxicokinetic parameters for plasma were calculated, such as.

$AUC_{0-\infty}$	Area under the plasma radioactivity concentration-time curve extrapolated from time 0 to infinity
$t_{1/2\ a}$	half-life of the absorption
$t_{1/2\ e}$	half-life of the elimination (terminal half-life)
C_{max}	maximum radioactivity concentration in plasma after single (first) dose administration
t_{max}	time at which the highest radioactivity concentration occurs in plasma after a single dose
MRT	mean residence time describing the sojourn (elimination and/or metabolism) of the chemical (or total radioactivity) in the body

The evaluation was performed with dose normalised values for the plasma concentrations. A standard two compartment disposition model was used for curve fitting computation.

7. Quantification and Identification of Metabolites

For metabolic profiling, the urine and faecal samples were pooled across individual animals for a given collection interval for each sex. Liquid chromatography (LC)/mass spectrometry (MS) methods were employed for identification and quantification of the metabolites in each sample. The identification of metabolites was mainly based on comparison of the urine profile with results of the organ metabolism study with [furanone-4-14C]BYI 02960 (Koester 2012b; Study No. M1824574-1). The minor metabolites or trace metabolites BYI 02960-iso-OH and BYI 02960-OH-SA were identified by HPLC comparison with urine samples or faeces extracts from the ADME study with [pyridinylmethyl-14C]BYI 02960 (Klempner., 2012; Study No. M1824559-4). The metabolite BYI 02960-difluoroethyl-amino-furanone was isolated from urine by repeated HPLC fraction and subjected to high resolution LC/MS analysis. Additional characterisation of the polar peak 1 (unknown 1) was performed by TLC analysis after isolation from urine.

In the Koester (2012b) organ metabolism study with [furanone-4-14C]BYI 02960 (Study No. M1824574-1), parent compounds and metabolites were identified by:

- Spectroscopic investigations (LC-MS) of the urine from males and females: BYI 02960-difluoroethyl-amino-furanone, isomers 1 and 3 of BYI 02960-OH-gluA, BYI 02960-OH and BYI 02960,

- HPLC comparison of the urine sample of males and females with the radiolabelled reference compounds BYI 02960-difluoroethyl-amino-furanone, and the isomers 1 and 3 of BYI 02960-OH-gluA,
- TLC-co-chromatography of urine of males and females with the radiolabelled reference compound BYI 02960-DFA,
- Comparison of the HPLC-radiochromatograms from all samples of each individual test, and
- Comparison of the HPLC-radiochromatograms from the specific samples of male and female urine.

Other peaks or peak groups additionally detected in the HPLC-profiles were designated as "unknown". They were characterised by their behaviour during extraction and clean-up and the retention times in the HPLC-chromatograms.

II. RESULTS AND DISCUSSION

A. STORAGE STABILITY

No stability data was reported for the test substance and metabolites in plasma and urine stored at <-18°C, nor in freeze dried samples including faeces stored at 4°C.

B. MAIN STUDY

Clinical Observation

There were no observable clinical signs of toxicity.

Recovery
Mean total radioactivity recovery was approximately 96% and 102% of the administered dose in males and females respectively. The slightly lower recovery for males was suggested to be due to a higher exhalation of radioactivity ($^{14}CO_2$) with expired air (i.e. in the autoradiography study with [furanone-4-^{14}C]BYI02960 (Koester, 2012b; Study No. M1824574-1) approximately 3% of the dose administered were detected in expired air of males and approximately 1% volatiles were found for females), which was considered unnecessary to determine due to the low proportion of radioactivity in expired air in the study it was measured (Koester, 2012a). For all dose groups, the majority of radioactivity was recovered from the urine, and together with the moderate recovery from faeces, excreta accounted for almost all of the recovered radioactivity (Table 3).

Table 3: Balance of radioactivity in excreta and in organs and tissues (Percent of total dose administered)

	2 mg/kg bw, oral	
	M, oral (group 1)	**F, oral (group 2)**
	Mean %	**Mean %**

Faeces	16.59±16.31	10.38±23.64
Urine	78.96±5.97	91.37±3.46
Sum of excreta	95.56±4.25	101.70±1.37
Body excluding GIT	0.476±9.76	0.171±17.67
GIT	0.017±19.82	0.011±7.15
Total Body	0.493±9.48	0.181±16.88
Balance	**96.05±4.28**	**101.90±1.34**

Data excerpted from pages 106 and 113, of the study report.

Absorption

The pharmacokinetic results indicated that absorption occurred immediately following administration of a single (2 mg/kg bw) oral gavage dose. Gastrointestinal absorption of the test substance was at least 79% in males and 91% in females, as >79% and >91%, respectively, of the total dose administered were detected in urines and in the carcass excluding GIT (Table 4).

Table 4: Time course of mean radioactivity measured in the plasma following a single oral dosing 2 mg/kg bw [furanone-4-^{14}C]BYI02960 to male and females (Equivalent concentration [mg a.s. equiv. /kg sample])

Time (hours)	Male		Female	
	Mean	CV[%]	Mean	CV[%]
0.17	0.2883	13.05	0.4562	9.72
0.33	0.7373	27.02	1.1720	22.39
0.67	1.2720	14.04	1.7870	11.55
1	1.4180	11.50	1.8570	8.10
1.50	1.4570	10.15	1.9120	6.31
2	1.4460	8.52	1.8610	5.01
3	1.3570	7.26	1.7410	6.97
4	1.2550	6.03	1.5880	9.04
6	1.0220	6.71	1.2990	12.06
8	0.8145	8.47	1.0140	15.18
24	0.0621	14.41	0.0515	22.89
28	0.0462	15.30	0.0326	22.23
32	0.0375	15.46	0.0264	16.99
48	0.0168	13.41	0.0091	27.99
52	0.0162	14.81	0.0079	21.99
56	0.0149	16.10	0.0076	6.30
72	0.0113	18.23	0.0062	22.91
96	0.0087	14.70	NC	NC
120	0.0066	14.61	NC	NC
144	0.0048	39.27	NC	NC
152	0.0048	11.78	NC	NC
168	NC	NC	NC	NC

NC – denotes mean values not calculated because more than one quarter of the individual values used to derive the mean were below the LOQ

Data excerpted from pages 109 and 116 of the study report.

For both sexes, plasma concentrations declined to approximately 50% of C_{max} within 8 hours and to approximately. 3 – 4% of Cmax within 24 hours. The concentrations declined further significantly after 24 hours, and fell below the limit of quantatation (LOQ) after 168 hours for males and after 96 hours for females. Male rats exhibited about twofold higher concentrations than females in the time range ≥48 hours (which was considered likely due to higher proportions of [furanone-4-14C]BYI 02960 metabolized to C1 and C2 compounds and incorporated in biomolecules or exhaled as $^{14}CO2$ in males compared to females). At the time of sacrifice, 168 hours after administration, the plasma concentrations were below LOQ for both male and female animals. Biokinetic modelling of mean values between 0 to 72 hours in females and 0 to 152 hours in males was undertaken, using a three compartment model to achieve a good fit due to the biphasic nature of the plasma curves. The corresponding concentration-time curve of the modelled plasma data is shown in Figure 1 and Figure 2.

Figure 1: Time course of total radioactive residues in plasma in males

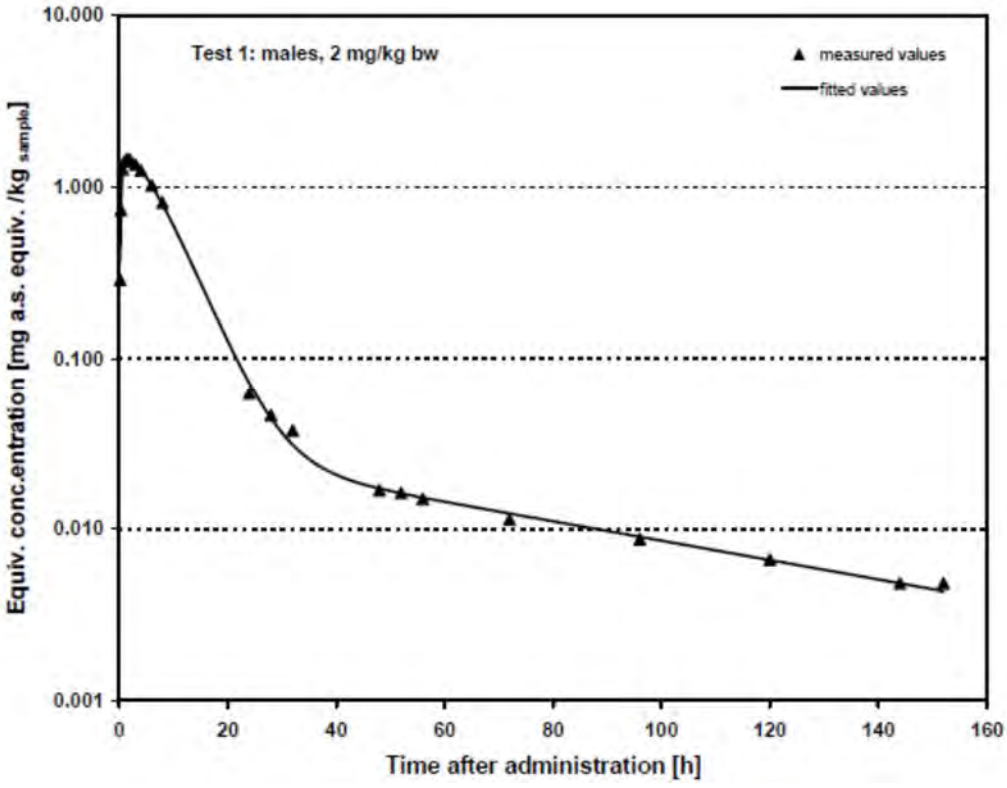


Figure excerpted from page 64 of the study report.

Figure 2: Time course of total radioactive residues in plasma in females

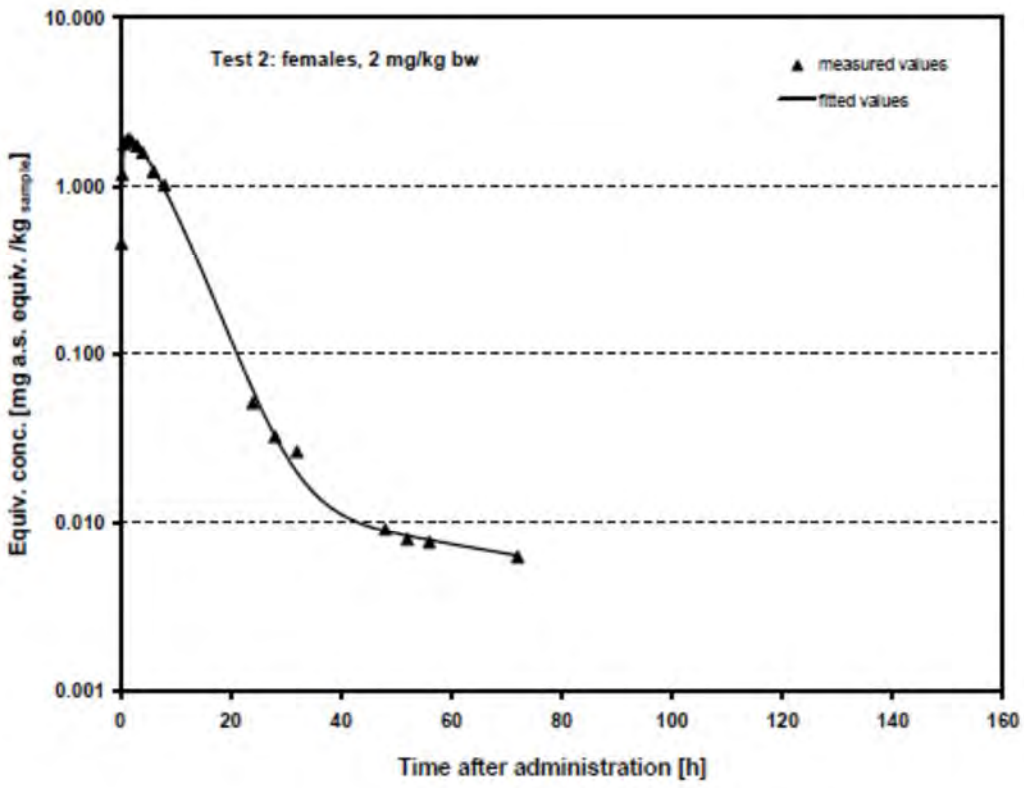


Figure excerpted from page 65 of the study report.

The AUC – extrapolated from 0 to affinity for both sexes was similar, at 16.0 and 18.2 mg/kg/h in males and females respectively. After an initial short first elimination half-life of approximately 3 hours which was attributed to the parent compound, there was a long second half-life of approximately 53 hours attributed to incorporation of the radiolabel into biomolecules. The mean residence time was 17.6 and 11.5 hours ni males and females respectively. A summary of the pharmacokinetic pararmeters are shown in Table 5.

Table 5: Pharmacokinetic parameters of [furanone-4-^{14}C]BYI02960 after oral dosing to male and females

Parameter	Male	Female
t_{max}[h] measured	1.50	1.50
t_{max}[h] modelled	1.60	1.34
C_{max}[µg/mL]measured	1.457	1.912
C_{max}[µg/mL]modelled	1.47	1.90
$t_{1/2abs}$[h]	0.232	0.166
$t_{1/2\ elim1}$[h]	3.07	2.88
$t_{1/2\ elim2}$[h]	53.1	53.6
$AUC_{0-\infty}$[mg/kg*h]	16.0	18.2
MRT[h]	17.6	11.5

Data excerpted from page 53 of the study report.

Distribution

A mean of approximately 0.5% of the total administered dose was detected in the body of male rats at the time of sacrifice 168 hours after oral dosing, of which a very minor amount of 0.02% was in the GIT. Similarly in females, approximately 0.2% of the total administered dose was detected in the body of male rats at the time of sacrifice 168 hours after oral dosing, of which a very minor amount of 0.01% was in the GIT. Mean residual concentrations in tissues demonstrated a wide distribution, and ranged from 0.0025 (plasma) to 0.0336 mg/kg sample (thyroid) in males, and ranged from 0.0012 (plasma) to 0.0131 mg/kg sample (thyroid) in females. Levels for most organs and tissues were very similar and in the range of approximately 0.005 to 0.01 mg/kg sample in males, and in the range of approximately 0.002 to 0.005 mg/kg sample in females (Table 6). This slight difference in mean residual concentrations in tissues (i.e. higher values I males compared to females) was considered likely caused by the higher rate of radioactivity incorporation in biomolecules for male rats.

Table 6: Radioactive residues in organs and tissues at sacrifice following a single oral administration of 2 mg/kg bw [furanone-4-14C]BYI 02960 to male and female rats (Equivalent concentration [mg a.s. equiv. /kg sample])

Organ/tissue	**Mean± CV[%]**	
	M	**F**
Blood_cells	0.0083±14.03	0.0038±13.99
Plasma	0.0025±18.44	0.0012±17.36
Carcass	0.0079±9.41	0.0031±15.60
Heart	0.0065±17.11	0.0029±12.86
Brain	0.0072±19.63	0.0033±16.29
Kidneys	0.0104±17.50	0.0045±13.26
Liver	0.0128±19.60	0.0081±22.29
GIT***	0.0033±21.27	0.0039±23.09
Gonads	0.0059±24.03	NA
Uterus	NA	0.0035±36.41
Adrenal_gland	0.0200±12.99	0.0114±30.64
Harderian_gland	0.0241±26.43	0.0091±20.84
Thyroid_gland	0.0336±21.65	0.0131*±27.08
Spleen	0.0081±17.54	0.0032±19.65
Lung	0.0075±16.69	0.0048±9.09
Eyes	0.0053±10.52	0.0048±9.34
Skin	0.0111±14.50	0.0047±31.19
Bone_femur	0.0085±15.09	0.0049±9.50
Fat_perirenal	0.0118±21.00	0.0058±42.81
Leg muscle	0.0069+17.75	0.0023+10.62
Average Conc.	**0.0106**	**0.0052**

Data excerpted from pages 111 and 118 of the study report.

Excretion

Excretion was rapid and almost complete by 168 hours post dosing with a mean of approximately 96% and 102% of total administered dose being excreted by this time point in males and females respectively, with the majority being excreted by 24 hours post dosing (90% and 97% respectively). The major route of excretion was urine, accounting for approximately 79% and 91% of the dose by in males and females respectively, with approximately 75% in males and 87% in females of total dose being excreted by this route within 24 hours. Faecal excretion accounted for approximately 18% and 10% of the total administered dose in males and females approximately, with approximately 15% in males and 9% in females being excreted within 24 hours (Table 7).

These results are in good accordance to the excretion behaviour detected in all other rat studies (Weber 2011a, Study no. M1824567-3; Klempner 2011, Study no. M1824559-4; Koester 2012a, Study no. M1824575-2; Koester 2012b, Study no. M1824574-1; Koester 2011b, Study no. M1821760-5; Koester 2011a, Study no, M1821837-0).

Table 7: Time course of cumulative excretion of radioactive residues via urine and faeces following a single oral dosing of 2 mg/kg bw [furanone-4-^{14}C]BYI02960 in male and female rats (Cumulative excretion in percent of total dose administered)

Time (hour)	**Mean ± CV[%]**	
	M	**F**
Faeces		
24	14.76±12.99	9.45±25.69
48	16.40±15.99	10.29±23.86
72	16.50±16.13	10.33±23.70
96	16.54±16.20	-
120	16.56±16.22	9.49±22.08
144	NC	NC
168	17.53±13.63	NC
Urine		
4	8.74±78.17	6.52±63.34
8	33.86±21.76	18.32±70.15
12	39.55±36.46	-
24	75.04±7.35	87.31±3.70
48	78.40±6.05	90.44±4.07
72	78.75±5.98	90.89±4.00
96	78.86±5.98	91.14±3.71
120	78.91±5.98	91.24±3.60
144	78.94±5.97	91.33±3.51
168	78.96±5.97	91.37±3.46
Sumtotal	**95.56±4.25**	**101.70±1.37**

NC – denotes mean values not calculated because more than one quarter of the individual values used to derive the mean were below the LOQ.
Data excerpted from pages 111 and 118 of the study report.

Metabolism

Urine

Over 48 hours, the main compound in urine was parent BYI 02960 which represented approximately 48% of the total dose administered in males and 70% in females. Only one major metabolite was detected, BYI 02960-OH, which accounted for approximately 14% of the dose in males and 11% in females. Other identified metabolites all present at <4% of the total administered dose were BYI 02960-difluoroethyl-amino-furanone (approximately 3% in males and 1% in females), BYI 02960 OH-gluA isomer 1 (approximately 1% in males and <1% in females) BYI 02960 OH-gluA isomer 3 (approximately 2% in males and 1% in females), BYI 02960 des-difluoroethyl (approximately 2% in males and 3% in females), and BYI 2960-OH-SA (<1% in both sexes). An unknown very polar metabolite fraction (peak 1) represented 5.22% of the dose in the urine of male rats and 2.42% in female rats. This peak was isolated from the urine pool sample 0-48h of male rats and analysed by TLC with radio detection. The sample could be separated into five compounds representing between 0.19% and 2.75% of the dose, but none of these could be identified. A number of other unknown metabolites were detected in males and females all present at <0.6% of the total administered dose (Table 8).

In total over 48 hours post dosing, approximately 79% of the total administered dose was excreted in urine of male rats of which approximately 71% were identified and approximately 8% characterised but not identified. In female rats approximately 90% of the administered total dose was excreted with urine of which approximately 86% were identified and approximately 4% characterised but not identified. With the exception of the polar metabolite (peak 1), all other characterised metabolites were found at levels of <0.6% of the total dose administered in both sexes (Table 8).

Faeces

Over 24 hours, the main component in faeces of male rats was the metabolite BYI 02960-OH which accounted for approximately 7% of the total administered dose. The parent compound BYI 02960 was present at approximately 6%. In contrast, in females the main component in faeces was the parent compound BYI 02960 which was present at approximately 6% while the metabolite BYI 02960-OH was present at approximately 3%. Other identified metabolites all present at <0.3% of the total administered dose were BYI 02960-difluoroethyl-amino-furanone (<0.1% in males only), BYI 02960 des-difluoroethyl (approximately 0.2% in males and 0.25% in females), BYI 2960-OH-SA (<0.1% in both sexes), and BYI 2960-iso-OH (approximately 0.25% in males and <0.1% in females). A number of other unknown metabolites were detected in males and females all present at <0.2% of the total administered dose (Table 8).

In total over 24 hours post dosing, approximately 15% of the total administered dose were excreted in the faeces of male rats of which approximately 14% were identified and approximately 0.4% characterised but not identified. In female rats approximately 9% the administered total dose were excreted in the faeces of which approximately 9.0% were identified and approximately 0.1% characterised but not identified. All other characterised metabolites were found at levels of <0.2% of the total dose administered in both sexes (Table 8).

Table 8: Percent of dose of parent compound and metabolites in urine and faeces

Sex		**Male**			**Female**		
Sample		**Urine**	**Faeces**	**Excreta**	**Urine**	**Faeces**	**Excreta**
Time [h]		**0-48**	**0-24**	**sum**	**0-48**	**0-24**	**sum**
% of dose administered		**78.40**	**14.76**	**93.16**	**90.44**	**9.45**	**99.89**
Peak ID	**Compound (report name) BYI 2960-**						
21	**Parent**	**48.34**	**6.34**	**54.68**	**70.02**	**5.94**	**75.96**
5	Difluroethyl-amino-furanone	3.42	0.07	3.49	0.96	---	0.96
9	OH-gluA (isomer 1)	1.13	---	1.13	0.49	---	0.49
12	OH-gluA (isomer 3)	2.17	---	2.17	1.01	---	1.01
14	Des-difluroethyl	1.87	0.24	2.12	3.09	0.26	3.35
19	OH-SA	0.16	0.09	0.24	0.25	0.09	0.34
20	OH	13.75	6.86	20.60	10.65	2.60	13.25
22	Iso-OH	---	0.26	0.26	---	0.07	0.07
Total identified		**70.83**	**13.86**	**84.70**	**86.46**	**8.96**	**95.42**
1	unknown 1 total	5.22	0.08	5.30	2.42	0.04	2.45
2	unknown 2	0.46	---	0.46	0.19	---	0.19
3	unknown 3	0.53	0.03	0.57	0.05	---	0.05
3a	unknown 4	---	0.03	0.03	---	---	---
4	unknown 5	0.40	---	0.40	0.12	---	0.12
6	unknown 6	0.05	---	0.05	0.16	---	0.16
7	unknown 7	---	0.02	0.02	0.07	0.02	0.09
8	unknown 8	---	0.03	0.03	0.06	0.05	0.11
10	unknown 9	0.24	---	0.24	0.44	---	0.44
11	unknown 10	0.02	---	0.02	0.02	---	0.02
13	unknown 11	0.19	0.06	0.25	0.23	---	0.23
15	unknown 12	---	---	---	0.14	---	0.14
16	unknown 13	0.40	0.13	0.53	0.02	---	0.02
17	unknown 14	0.04	0.06	0.10	0.06	---	0.06
18	unknown 15	0.02	---	0.02	---	---	---
Total characterised		**7.57**	**0.43**	**8.00**	**3.98**	**0.11**	**4.08**
Total accounted		78.40	14.30	92.70	90.44	9.07	99.51
Solids		---	0.45	0.45	---	0.37	0.37
Losses		---	0.01	0.01	---	<0.01	<0.01
Samples not analysed		0.56	1.83	2.39	0.93	0.93	1.86
Total		**78.96**	**16.59**	**95.55**	**91.37**	**10.38**	**101.75**

Note: Samples not analysed were urine 48-72 h, 72-96 h, 96-120 h, 120-144 h, and 144-168 h; faeces 24-48 h, 48-72 h, 72-96 h, 96-120 h, 120-144 h, and 144-168 h.

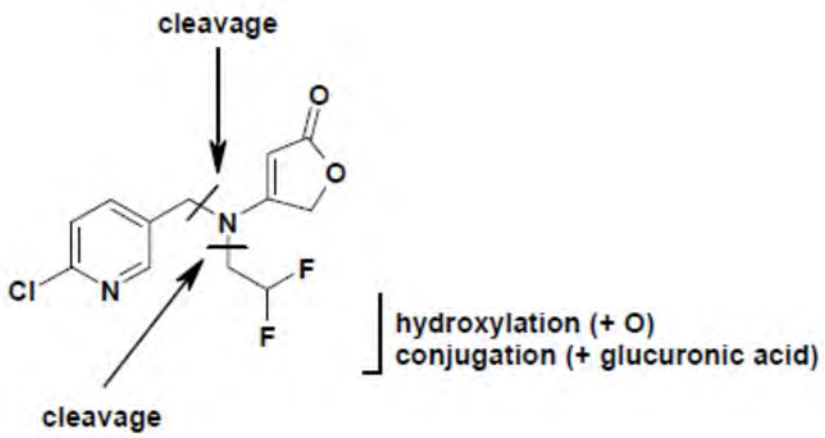
Data excerpted from pages 56 and 58 of the study report (Note: values in urine 0-4 h, 4-8 h, 8-12 h, 12-24 h and 24-48 h are not included in Table 8)

Overall, BYI 02960 was moderately metabolised to approximately 20 metabolites. Parent compound represented the predominant part of the radioactivity in urine but in faeces samples the metabolite BYI 02960-OH was more prominent. All other identified and characterised metabolites represented a minor part of the dose. The metabolic profiles in urines and faeces were very similar for both sexes but male rats exhibited a higher rate of metabolite formation compared to female animals.

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

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

The following figure shows schematically the positions in the molecule, which are involved in the metabolic reactions:



The proposed biotransformation pathway of [furanone-4-^{14}C] BYI02960 is presented in Figure 3 below.

Figure 3. Proposed metabolic pathway of [furanone-4-^{14}C] BYI02960

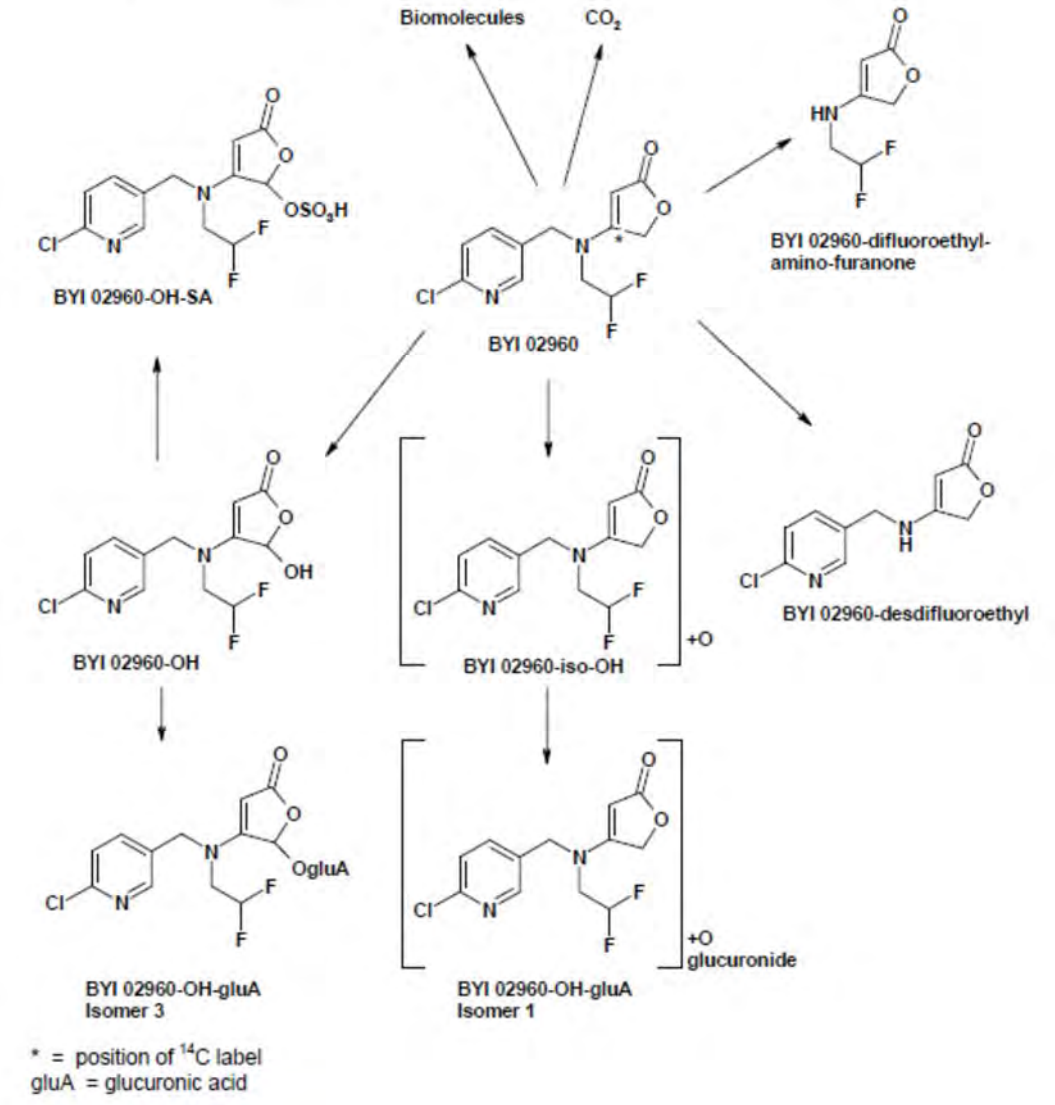


Figure excerpted from page 90 of the study report.

III. CONCLUSION

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

- The test compound was nearly completely absorbed since >79% of the administered dose was detected in the urine and the body without GIT at sacrifice.
- The distribution of the radioactivity within the body was rapid and extensive, and the maximum plasma level (C_{max}) was reached within 1.5 hours after administration for both male and female rats. From the maximum, the radioactivity level declined slowly

down to approximately 50% of C_{max} after 8 hours and to <1% of C_{max} after 48 hours after dosing.

- Excretion of BYI 02960 related radioactivity was fast and occurred mainly by the renal route. Female rats exhibited a slightly higher renal excretion rate of approximately 91% than males with approximately 78%.
- The major part of the dose (>89% for males and >96% for females) was excreted within 24 hours after treatment. But excretion was ongoing in the time after 24 hours.
- At the time of sacrifice, 168 hours after administration, a small proportion of approximately 0.5% of the dose administered was still detected in the body without GIT of male rats and approximately 0.2% in the ones of females. The residue in plasma was 0.0025 mg/kg for males and 0.0012 mg/kg for females. For all organs and tissues levels were <0.04 mg/kg in males and <0.02 mg/kg for females.
- Parent compound, one major (BYI 02960-OH) and six minor metabolites were identified in urine and faeces samples. Identification rates were >90% of the total radioactivity in urine and > 90% of the total radioactivity in faeces. Parent compound represented the predominant part of the radioactivity in urine but in faeces samples the metabolite BYI 02960-OH was more prominent.
- Approximately 85% of the total dose administered was identified in excreta of male rats and approximately 95% in excreta of females. Another approximate 8% of the dose in males and approximate 4% in females were characterised by their chromatographic behaviour. All metabolites representing >1% of the dose administered were identified with the exception of a very polar fraction which may contain biomolecules formed by complete oxidative degradation of the furanone ring to C1 and C2 compounds.
- The metabolic transformation of BYI 02960 was principally oxidative and took place at least at 3 different structural positions of the test compound.
- Basically, male rats showed a higher rate of metabolism with only approximately 55% of unchanged parent compound found in excreta whereas 76% of unchanged BYI 02960 were found in the excreta of female rats. But the metabolic patterns were very similar in males and females.
- Male rats also exhibited a higher proportion of the polar metabolite fraction and higher residues in the body at the time of sacrifice, as well as a higher proportion of radioactivity in exhaled air in a study using [pyridinylmethyl-^{14}C]BYI 02960 (Koester, 2011b; Study No. M1821760-5). All these observations suggest that the furanone-4-^{14}C radiolabel is not completely stable and that a small part of the dose obviously underwent extensive biotransformation to C1- and C2-fragments that resulted in an incorporation of radioactivity into biomolecules, in particular in male rats.
- The metabolic pattern was in good accordance with that obtained from the corresponding organ metabolism rat study using [furanone-4-^{14}C]BYI 02960 (Koester, 2012b; Study No. M1824574-1).

Based on the results obtained the absorption, distribution, excretion, and metabolism of [furanone-4-^{14}C]BYI 02960 in rats are assumed as adequately understood.

Primary review: Australia
Secondary review: USEPA

IIA 5.1.1/07 Whole body distribution study in rats: [furanone-4-^{14}C]BYI 02960

Report: Koester, J (2011b) Quantitative whole body autoradiography of [furanone-4-^{14}C]BYI 02960 in male and female rats: distribution of total radioactivity and elimination from blood, organs and tissues after single oral administration including determination of radioactivity in the excreta and exhaled $^{14}CO_2$. Bayer CropScience AG, Development - Environmental Safety, Metabolism/ADME and Environmental Fate, D-40789 Monheim am Rhein, Germany. Study No. M1821760-5. May. MRID 48844144. Unpublished.

Guidelines: US EPA 870.7485 (1998)
OECD TG 417 (2010)
JMAFF 12 Nousan 8147 (2001 amendment)
PMRA Dir2005-01: Section 11 (2005)
EC No 1107/2009 (2009)

Deviations: None

GLP: Yes. Signed and dated GLP, Quality Assurance and Data Confidentiality statements were presented in the report.

Executive summary:

In a quantitative autoradiography kinetic study, 8 male and 8 female Wistar rats were administered by oral gavage a single dose of 5 mg/kg bw [furanone-4-^{14}C]BYI 02960 in 0.5% aqueous Tragacanth®. The test compound was radiolabelled with ^{14}C in the C4 position of the furanone ring of the molecule. A single animal of each sex was sacrificed by carbon dioxide asphyxiation at selected time points from 1 hour to 168 hours post dosing, and urine and faeces samples were also taken up to sacrifice along with carbon dioxide for selected time periods and the amounts of radioactivity in the samples determined. The distribution of total radioactivity to and elimination from blood, organs and tissues were also analysed qualitatively and quantitatively in sacrificed animals by whole-body autoradioluminography. The control animals (a single male and female) were sacrificed at 4 hours, for detection of possible chemo-graphic effects, and demonstrated no such effects.

BYI 02960 was readily absorbed from the gastrointestinal tract and distributed throughout the body immediately after administration. The excretion of radioactivity via urine and faeces was almost completed after 2 days with renal excretion as the predominant route. For male rats, by 168 hours post dosing approximately 81% in males and 88% in females of the administered dose were recovered in urine and approximately 14% in males and 6% in females in the faeces. From approximately 1 % (females) to 3% (males) of the dose was exhaled as ^{14}C-carbon dioxide during a sampling period of 48 hours. This demonstrated that for a small portion of the dose the furanone ring of the molecule obviously underwent extensive biotransformation to

C1- and C2-fragments and the terminal product $^{14}CO_2$. Excretion was nearly complete approximately 2 days after administration.
In male and female rats, maximum TRR-values (= CEQmax: expressed as µg a.s. equiv. /g) were reached for nearly all organs and tissues at one hour after administration (= tmax). At this time, the values for liver, kidney, brown fat, myocardium, nearly all glandular and hormonal organs, and the olfactory bulb were higher than in blood in males, and in the liver, kidney, myocardium, nearly all glandular and hormonal organs, and the olfactory bulb in females, suggesting a preferred clearance from blood and distribution to those organs that are responsible for metabolism, excretion and secretion. Very low levels were found in the brain, spinal chord and in renal fat in both sexes. At day 7, the comparatively high TRR-value still seen in the nasal mucosa in both sexes is considered directly related to the exhalation of ^{14}C-carbon dioxide.

From the peak values, the concentrations declined with a biphasic kinetics. The second slow decline phase that started after 24 hours in males and after 48 hours in females is probably linked to small carbon units (C1- or C2-fragments) that entered the carbon pool of endogeneous compounds used for example for the biosynthesis of protein and fat.

After seven days, low radioactive residues were measured in nearly all organs and tissues of the rats due to the before mentioned incorporation of C1- or C2-fragments into the endogeneous carbon pool. The terminal residues were always by a factor of 1.4 to 4.7 higher in males than in females. A similar ratio of approx. 3 was also found for the formation of 14C-carbon dioxide in males compared to females. However, the results of this study do not provide evidence for accumulation or significant retention of [furanone-4-^{14}C]BYI 02960 in male and female rats.

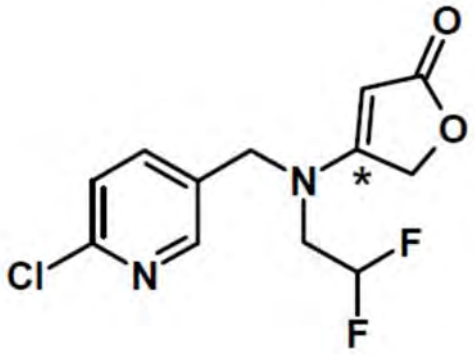
Sex related quantitative differences in metabolism leading to more C1- and C2-fragments and also higher incorporation of these components into the endogenous carbon pool in males are presumably responsible for this observation.

It was also demonstrated that there was sufficient stability of the unchanged parent compound in plasma following deep frozen storage at ≤ -18 °C for up to approximately 31 months.

This study is fully reliable (acceptable/guideline) and satisfies the study requirements of a toxicokinetics study, albeit through use of an atypical method of radioactivity detection and quantification (U.S. EPA 870.7485; OECD Section 4 Part 417; JMAFF 12 Nousan 8147; PMRA Dir2005-01: Section 11; EC No 1107/2009).

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	BYI02960
	Batch #'s:	BYI 02960-PU-01 or (for females) BYI 02960-PU-02
	Purity:	99.9% for BYI 02960-PU-01; 99.4% for BYI 02960-PU-02 (MS, NMR)
	Description:	Beige powder
	CAS #:	951659-40-8
	Stability of test compound:	Stated to be stable for two years if stored at 5±5°C (assay date 16 Jan 2008).
2.	Radiolabeled test material:	[furanone-4-^{14}C]BYI02960
	Lot/Batch #:	KATH 6109 or (for females) KATH 6252
	Chemical structure /positions of radiolabel:	
		* Denotes position of radiolabel
	Radio-chemical purity:	>99% (HPLC UV, TLC)
	Specific activity:	3.94 MBq/mg (30.73 Ci/mol)
	Description:	Solid, dried in vacuum
	Stability of test compound:	The positions of the radiolabels are considered to be stable. Stated to be stable at -18°C over the duration of the study.
3.	Vehicle and/or positive control:	0.5% aqueous Tragacanth®
4.	Test animals	
	Species:	Rat
	Strain:	Wistar Hsd/Cpb: WU
	Age at dosing:	8 weeks (males), 9 weeks (females)
	Weight at dosing:	196 to 222 (males), 190 to 208 (females)
	Source:	Harlan Netherlands BV, Kreuzelweg 53, NL-5960 AD Horst, Netherlands.

Acclimation period:	Seven days in Makrolon® metabolism cages prior to dosing.
Diet:	Rat/mice maintenance long life diet No. 3883.0.15, (Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland), *ad libitum* while in metabolism cages, except for approx. 16 hours prior to, and for 8 hours after dosing.
Water:	Tap water, *ad libitum*
Housing:	Individually housed in Makrolon® metabolism cages with wood floor shavings until termination. Cages permitted an 'almost' quantitative separate urine and faeces collection.

5. Environmental conditions

Temperature:	22 to 24°C
Humidity:	45 to 60%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

6. Preparation of the dosing solutions

Rats were dosed with [furanone-4-^{14}C]BYI02960. The [furanone-4-^{14}C]BYI02960 radiolabel was diluted with technical BYI02960. The dose vehicle 0.5% aqueous Tragacanth® was added using an ultrasonic bath and stored at 5ºC until use and kept homogenous by magnetic stirring bars and glass balls. Dose route, dose level, volume, and radiolabel data are presented in Table 1. The test compound was administered by a single oral gavage dose.

Table 1. Dose Levels, Volumes, and Radiochemical Data

Test No.	**Administration suspension**							
	Total prepared		**Administered per rat**					
	Amount (mg)	**Volume (mL)**	**Volume/ Amount**	**Target dose (mg/kg bw)**	**Radioactivity (dpm)**	**Amount (mg)**	**Actual dose (mg/kg bw)**	**Mean rat weight (g)**
1 (M)	9	18	2.0 mL /1.0 mg	5	291, 717, 031	1.23	5.71	216
2 (M)	12	24	2.0 mL /1.0 mg	5	250, 172, 307	1.06	5.25	202
3 (F)	12	24	2.0 mL /1.0 mg	5	231, 680, 392	0.98	4.97	197

Note: The study also included a control male and female rat which did not receive the radiolabelled test material

Data excerpted from page 21 of the study report.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

   10 April 2010 to 18-Nov-2010

2. Main study

   The basis for the dosing regimen of 5 mg/kg bw was not reported. Animals were assigned to test groups according to Table 2.

Table 2. Study design

Group	No. of animals	Achieved oral dose (mg/kg bw)	Sampling intervals or timepoint (hours)				
			Urine	Faeces	Expired Air*	Plasma	Whole body including whole blood
1	5 males	5.71	-	-	-	10 min, 30 min, 1, 2, 7, 24 (at time of sacrifice)	-
2	8 males	5.25	0 – 1, 1 – 4, 4 – 8, 8 – 24, 24 – 48, 48 – 72, 72 – 96, 96 – 120, 120 – 144, 144 – 168	0 – 24, 24 – 48, 48 -72, 72 – 96, 96 – 120, 120 – 144, 144 – 168	0 – 24, 24 – 48	-	1, 4, 8, 24, 48, 72, 120, 168 (at time of sacrifice)
3	8 Females	4.97					

*Carbon dioxide from expired air was collected from 4 animals in Group 1 and Group 2

Note: A single animal from Group 1 was sacrificed at each of the times given Group 1 animals and a plasma sample collected. A plasma sample from the animal sacrificed 2 hours post dosing was stored deep frozen at ≤ -18 °C for up to approximately. 31 months. After 31 months the stability of unchanged parent compound was determined by HPLC. No urine, faeces or expired air samples or whole body sampling was undertaken in these animals.

It is also reported that for Group 1 animals that liver, kidneys, testes and muscle samples were dissected from all animals at sacrifice and . Urine samples were collected from the animals of 1, 2, 7 and 24 hours and a faeces sample from the animal of 24 hours. Furthermore, that the urine samples were analysed directly by HPLC and the faeces and organ samples after extraction using conventional methods. However, it was stated that the results from all these samples and are not reported because finalised data are compiled and addressed in the respective rat ADME guideline study (Klempner, 2011; Study No. M1824560-6) and in an organ metabolism study (Koester, 2011b; Study No. M1824559-4) using [furanone-4-^{14}C]BYI02960.

A single animal from Group 2 and Group 3 was sacrificed at each of the times given for whole body sampling.

Control animals (a single male and female not shown in the table above) were sacrificed at 4 h, and no urine, faeces or expired air samples were obtained from these animals. The control animals were used for detection of possible chemographic effects.

3. Tissue Distribution at sacrifice

At the designated sacrifice time (see Table 2 above) animals in each group including control animals were sacrificed by carbon dioxide asphyxiation. Sacrificed animals were immediately deep frozen in a solid carbon dioxide/ dichloromethane mixture at approximately -70 °C and embedded in aqueous carboxymethylcellulose (CMC) gel (7-8%). Sagittal sections (from cranial to caudal) were cut with a thickness of 50 µm at ca. -25 °C using a cryomicrotome, slightly sprayed with a liquid plaster to stabilise the whole section and dehydrated for at least 24 hours. Four to five sections showing the relevant organs and tissues of the respective body planes (e.g. eye-kidney) were prepared from each animal. The distribution of total radioactivity (i.e. without differentiation of unchanged test compound and radiolabelled biotransformation products) in rat organs and tissues was then determined qualitatively at the various time-points by whole-body autoradioluminography employing the Fuji BAS 5000® phosphorimaging system.

4. Quantitation of Radioactive Residues

In groups 2 and 3, radioactivity in plasma or blood cells could not be determined separately due to the autoradiographic sampling technique, and therefore only whole blood concentrations were determined. The measurement of the radioactivity in liquid samples (which included funnel rinses from the Makrolon® metabolism cage for urine samples) was carried out by LSC. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

Whole-body radiography was used to quantify radioactivity in a given tissue or organ. This method allows visualisation and quantitative evaluation of selective enrichments of radioactivity, particularly in certain parts of organs or tissues of the animal. The quantification of equivalent concentrations in the organ and tissues was performed using ^{14}C-spiked blood standards for calibration, with the radioactivity concentration of this standard being determined by LSC.

II. RESULTS AND DISCUSSION

A. STORAGE STABILITY

The chromatographic patterns of deep frozen stored plasma samples were determined at 2, 6, 18 and 31 months and seen to be similar, with the unchanged parent compound amounting to approximately 92% to 98 % without a clear trend for degradation over time. These results confirmed a sufficient stability of the test substance during storage in plasma.

No other storage stability data were provided.

B. MAIN STUDY

Clinical Observation

Observation of animals for clinical signs was not undertaken.

Absorption and distribution

Gastrointestinal absorption of the test substance was rapid with extensive distribution. In both sexes, maximum equivalent concentrations (tmax) were reached in nearly all organs and tissues at the first sampling time of 1 hour post-dosing. At tmax in both sexes, distribution was seen to be greatest towards those organs or tissues responsible for metabolism (liver, tissue/blood concentration value 1.76 in males, 1.83 females), excretion (kidney, 1.70 males, 1.64 females), secretion (e.g. adrenal, 1.92 males, 1.87 females, thyroid (1.38 males, 1.42 females), Harderian (1.38 males, 1.47 females) and salivary glands (1.34 males, 1.35 females) and to the organ which had contact to expired air (olfactory bulb 1.49 males). The lowest values were measured for seen in perirenal fat (0.14 males, 0.15 females), spinal cord (0.42 males, 0.34 females) and brain (0.35 males, 0.32 females).

The equivalent concentrations in blood, organs and tissues in both sexes declined rapidly with an approximately first order kinetics in the time range between 1 and 24 hours in males, and a slower decline from 24 to 168 hours, and in females a fast period between 1 and 48 hours and a slower decline or more or less stable period from 48 to 168 hours (i.e. biphasic kinetics in both sexes).

At 168 hour post dosing in both sexes, the equivalent concentrations in nearly all organs and tissues were still higher than in blood in both sexes. Higher tissue/blood concentration ratios were in a range between 1.05 (perirenal fat) and 9.87 (nasal mucosa) in males, and for females were in a range between 1.31 (uterus) and 18.36 (nasal mucosa). The relatively lowest values were seen in the skeletal muscle (0.84) and lung (0.76 in males), and bone marrow (0.99) and perineal fat (0.15) in females. However, at 168 hours, CEQmax had declined significantly, and were down to approximately 0.4% and 0.2% in blood in males and females respectively, 0.9% and 0.7% in nasal mucosa in males and females respectively, and approximately 3%, 3% and 2% in perirenal fat, spinal cord and brain in males. In all other organs and tissues, CEQmax was down to approximately 0.3% - 0.9% in males and 0.1% - 0.7% in females.

However, while relatively high values were seen in some organs and tissues at 168 hours post dosing, this corresponded to very low absolute levels of ≤ 0.05

mg/kg sample in males and ≤ 0.03 mg/kg sample in females, apart from the nasal mucosa (0.164 and 0.117 mg/kg sample in males and females respectively (Table 3).

Since no blackening of typical animal structures could be observed in control animals, chemo-graphic effects were excluded for the imaging data obtained for the sections of the animals treated with the radiolabelled test compound.

Table 3. Individual equivalent concentrations of radioactivity in blood, organs and tissues (equivalent concentration CEQ [µg a.s. equiv. /g])

Organ/Tissue	Male animal No.								Female animal No.							
	612	613	614	615	616	617	618	619	776	777	778	779	780	781	782	783
	Time of sacrifice (hours post dosing)															
	1	4	8	24	48	72	120	168	1	4	8	24	48	72	120	168
Blood	**3.896**	2.737	1.634	0.086	0.039	0.039	0.024	0.017	**4.250**	2.587	1.341	0.061	0.009	0.007	0.006	0.006
Liver	**6.860**	4.734	2.976	0.303	0.168	0.150	0.080	0.036	**7.757**	4.648	2.705	0.143	0.024	0.018	0.011	0.009
Renal cortex	**5.743**	4.346	2.247	0.146	0.056	0.059	0.035	0.021	**5.937**	3.373	2.027	0.094	0.015	0.010	0.008	0.008
Renal medulla	**7.529**	6.423	3.037	0.205	0.071	0.075	0.042	0.027	**7.971**	5.223	3.525	0.156	0.018	0.015	0.012	0.011
Kidney total	**6.636**	5.384	2.642	0.176	0.063	0.067	0.038	0.024	**6.954**	4.298	2.776	0.125	0.016	0.012	0.010	0.009
Brown fat	**4.286**	2.554	1.665	0.145	0.110	0.085	0.059	0.030	**3.604**	2.221	0.938	0.089	0.018	0.019	0.010	0.008
Perirenal fat	**0.552**	0.418	0.258	0.033	0.020	0.012	0.031	0.018	**0.654**	0.365	0.179	0.014	<LOQ	<LOQ	<LOQ	<LOQ
Skeleton muscle	**3.733**	2.896	1.426	0.073	0.026	0.029	0.018	0.014	**4.498**	2.441	1.398	0.058	0.006	<LOQ	<LOQ	<LOQ
Myocardium	**5.274**	3.544	2.131	0.097	0.037	0.035	0.026	0.016	**5.606**	3.174	1.826	0.075	0.009	0.006	0.006	0.006
Lung	**2.794**	2.555	1.250	0.077	0.030	0.028	0.017	0.013	**3.714**	1.997	1.036	0.050	0.006	0.005	0.004	<LOQ
Spleen	**3.754**	2.878	1.464	0.103	0.056	0.057	0.035	0.019	**4.238**	2.273	1.313	0.068	0.013	0.010	0.007	0.007
Pancreas	**4.960**	3.144	2.003	0.117	0.044	0.046	0.026	0.016	**5.546**	3.081	1.691	0.082	0.010	0.006	0.005	---
Bone marrow	**3.320**	2.395	1.309	0.182	0.104	0.081	0.041	0.019	**3.187**	2.295	1.002	0.075	0.019	0.013	0.008	0.006
Gonads	**2.493**	2.066	1.229	0.080	0.044	0.042	0.030	0.021	**4.143**	2.246	1.447	0.075	0.012	0.011	0.006	<LOQ
Brain	**1.372**	1.055	0.715	0.081	0.066	0.060	0.052	0.033	**1.376**	0.811	0.462	0.023	0.007	0.007	0.006	0.009
Uterus	NA								**4.305**	2.670	1.414	0.068	0.018	0.013	0.010	0.008
Spinal cord	**1.624**	1.253	0.788	0.108	0.080	0.079	0.067	0.042	**1.464**	0.941	0.495	0.029	0.009	0.009	0.008	0.011
Pituitary gland	**4.639**	2.890	1.766	0.123	0.077	0.073	0.040	0.026	**4.838**	2.950	1.516	0.076	0.012	---	---	---
Pineal body	**3.763**	2.534	1.506	0.105	---	0.048	0.031	0.014	**3.176**	2.444	1.244	0.053	---	---	---	---
Adrenal gland	**7.487**	4.793	2.914	0.358	0.143	0.131	0.072	0.039	**7.927**	4.739	2.498	0.172	0.057	0.036	0.021	0.026
Thymus	**3.869**	2.635	1.587	0.140	0.106	0.097	0.045	0.022	**4.287**	2.577	1.319	0.072	0.021	0.019	0.010	0.007
Thyroid gland	**5.391**	3.848	2.280	0.304	0.253	0.174	0.080	0.036	**6.024**	3.585	1.806	0.127	0.050	0.032	0.026	0.026
Salivary gland	**5.224**	3.691	2.237	0.138	0.063	0.059	0.037	0.020	**5.732**	3.450	1.833	0.085	0.013	0.010	0.007	0.006
Nasal mucosa	**1.943**	1.880	1.727	0.592	0.419	0.401	0.179	0.164	**1.689**	1.622	0.751	0.390	0.250	0.203	0.160	0.117
Vitreal body	1.785	**1.875**	1.301	0.197	0.091	0.059	0.049	0.015	**1.831**	1.615	1.110	0.212	0.041	0.032	0.013	0.009
Harderian gland	**5.384**	4.421	---	0.443	0.367	0.313	0.090	0.050	**6.238**	3.491	2.034	0.120	0.021	0.011	0.014	---
Olfactory bulb	**5.823**	4.097	2.875	0.547	0.434	0.371	0.128	0.052	---	---	**2.393**	0.194	0.068	0.064	0.021	0.017

Note: Maximum CEQ - values (= CEQmax) are shown in bold (1 hour was found to be CEQ_{max} for all tissues and blood, with the exception of the vitreal body in males where it was 4 hours, and the olfactory bulb of females, where it was 8 hours. **–** denotes that organ or tissue was visible in the rat sections but not discernible in the radioluminograms.

Data excerpted from pages 37 and 41 of the study report.

Table 4. Cumulative excretion of radioactivity in urine, faeces and exhaled air of rats

	Percent of radioactive dose administered															
	Male - Animal No.								**Female - Animal No.**							
	785	**786**	**787**	**788**	**789**	**790**	**791**	**792**	**794**	**795**	**796**	**797**	**798**	**799**	**800**	**801**
	Time of sacrifice (hours)															
	1	**4**	**8**	**24**	**48**	**72**	**120**	**168**	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
	Exhaled air															
24					2.74	2.54	1.71	2.33					0.50	0.63	0.66	0.82
48					3.25	3.01	2.02	2.83					0.58	0.71	0.77	0.96
	Urine															
1	4.30								3.82							
4		13.29	4.49	6.27	3.44	3.56	6.76	19.80		32.45	35.72	38.42	36.43	42.56	28.90	35.24
8			41.15	41.14	27.44	28.12	48.81	50.47			60.08	54.58	53.23	48.11	35.08	54.17
24				45.15	73.40	69.05	74.38	77.72				87.30	54.81	90.78	76.99	84.22
48					75.77	71.95	77.50	80.11					57.56	92.71	80.60	86.19
72						72.26	77.87	80.39						92.89	81.22	86.49
96							78.00	80.48							81.60	86.66
120							78.09	80.52							82.10	87.88
144								80.56								87.98
168								80.59								88.04
	Faeces															
24	*****	*****	*****	41.20[A]	13.54	13.41	10.80	12.76	*	*	*	6.57	33.30	4.32	4.94	4.69
48					15.09	15.48	13.07	13.68					34.84	5.18	6.46	5.60
72						15.84	13.30	13.76						5.22	6.58	5.75
96							13.36	13.79							6.73	5.79
120							13.39	13.80							6.76	5.81
144								13.82								5.83
168								13.83								5.84
Sum total	**4.30**	**13.29**	**41.15**	**86.35**	**93.69**	**91.35**	**94.50**	**96.44**	**3.82**	**32.45**	**60.08**	**93.87**	**92.98**	**98.82**	**89.54**	**94.84**

* – denotes faeces not collected.

Data excerpted from page 40 and page 44 of the study report.

Excretion

Over the study period, in both sexes the major part of the total dose administered was excreted in the urine (approximately 81% in males, 88% in females) and a minor part in faeces (approximately 14% in males, 6% in females), and a smaller amount exhaled over a 48 hour period from 48 hours post dosing (approximately 2 – 3% in males and 0.5 – 1% in females). This demonstrated that for a small portion of the dose the furanone ring of the molecule obviously underwent extensive biotransformation to C1-end C2-fragments and the terminal product $^{14}CO_2$ in males, with an even smaller portion undergoing biotransformation in females.

Thus, it can be seen that females exhibited a slightly higher renal excretion rates than males. Furthermore, excretion was rapid, with approximately 86% in males and 94% in females of the total administered radioactivity excreted by 24 hours post dosing, and the excretion nearly complete approximately 2 days after administration (Table 4).

III. CONCLUSION

BYI 02960, labelled with ^{14}C in the C4-position of the furanone ring of the molecule, was readily absorbed from the gastrointestinal tract of male and female Wistar rats after single oral administration. Test compound related radioactivity was distributed throughout the bodies immediately after administration however with a clear preference to those organs or tissues that are responsible for metabolism, excretion and secretion.

Peak concentrations of radioactivity for nearly all organs and tissues were reached at the first sampling point of one hour after dosing. From the peak values, the concentrations declined with a biphasic kinetic. The second slow decline phase that started after 24 hours in males and after 48 hours in females is probably linked to small carbon units (C1- or C2-fragments) that entered the carbon pool of endogeneous compounds used for example for the biosynthesis of protein and fat.

The radioactivity was quickly excreted, mainly by the renal route with a minor part in the faeces. The detection of ^{14}C-carbon dioxide in the exhaled air during a sampling period of 48 hours (up to approximately 3% in males and 1% in females) demonstrated that for a small portion of the dose the furanone ring of the molecule obviously underwent extensive biotransformation to C1-and C2-fragments and the terminal product $^{14}CO_2$. Over the 168 hour study period, a slightly higher proportion of renal excretion was seen in females (approximately 88%) compared to males (approximately 81%. Greater than 85% and 90% of the administered dose was excreted within 24 hours in males and females respectively, and excretion was nearly complete approximately 2 days after excretion (approximately 93 -94 in both sexes).

At the end of the tests (day 7), low radioactive residues were measured in nearly all organs and tissues of the rats due to the before mentioned incorporation of C1- or C2-fragments into the endogenous carbon pool. The terminal residues were always by a

factor of 1.4 to 4.7 higher in males than in females. A similar ratio of approximately 3 was also found for the formation of ^{14}C-carbon dioxide in males compared to females.

Sex related quantitative differences in metabolism leading to more C1- and C2-fragments and also higher incorporation of these components into the endogeneous carbon pool in males are presumably responsible for this observation. However, the results of this study do not provide evidence for accumulation or significant retention of [furanone-4-^{14}C]BYI 02960 in male and female rats.

Primary review: Australia
Secondary review: US EPA

IIA 5.1.1/05 Metabolism study in rats: [Furanone-4-^{14}C]BYI02960

Report: Koester J (2012b) [Furanone-4-14C]BYI02960 – Metabolism in Organs and Tissues of Male and Females. Bayer CropScience AG, Development - Environmental Safety, Metabolism/ADME and Environmental Fate, D-40789 Monheim am Rhein, Germany. Study No. M1824574-1. September. MRID 48844145. Unpublished.

Guidelines: US EPA 870.7485 (1998)
OECD TG 417 (2010)
JMAFF 12 Nousan 8147 (2000)
Deviations: None

GLP: Yes. Signed and dated GLP, Quality Assurance and Data Confidentiality statements were presented in the report.

Executive summary:

In a metabolism study, 4 male and 4 female Wistar rats were administered by oral gavage a single dose of 3 mg/kg bw [furanone-4-^{14}C]BYI02960 in 0.5% aqueous Tragacanth® and sacrificed by exsanguination under anaesthesia at 6 hours post dosing. The test compound was radiolabelled with ^{14}C in the C4-position of the furanone ring of the molecule. The metabolism and total radioactivity which included the test compound and metabolites was determined in urine samples for the time period 0 – 6 h as well as in plasma, liver, kidney, muscle (leg) and fat (perirenal) at the 6h sacrifice. Samples were analysed, and parent compound and metabolites identified, by radiochromatographic (HPLC, TLC) and spectroscopic (LCMS) methods.

Mean total radiolabel recovery was 100.60% of the administered dose in males, and 98.09% in females. Over the 6h duration of the study, urinary excretion of the administered dose was 1.17-fold higher in females than in males (36.64% and 42.82% respectively).

Highest radioactivity levels were detected in the liver (approx. 2.9 mg/kg for both sexes) and kidney (approx. 2.7 mg/kg for males and 4.3 mg/kg for females) which are the main organ responsible for metabolism and urinary excretion. The residue-values for plasma and the other tissues were comparable for both sexes and ranged from approx. 0.6 mg/kg for the perirenal fat up to approx. 1.5 mg/kg for the leg muscle.

BYI02960 was metabolised to a comparatively minor extent. The majority of metabolic components were identified in both sexes (approximately 89 – 100% of total radioactive residue (TRR) in plasma, and in extracts of organs and tissues as well as ≥ 88% of radioactivity in urine samples). Metabolic reactions took place at least at 3 different structural positions of the test compound. The principal metabolic reactions of [furanone-4-^{14}C]BYI 02960 in rats were: (1) hydroxylation followed by conjugation with glucuronic acid, (2) cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl and (3) cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

In the 6 h samples of plasma, and organs and tissues BYI 02960 was by far the largest component accounting for more than 72% of the total radioactive residues (TRR). For all identified metabolites, the values were less than 12% of the TRR. In the respective urine sample pools, the parent compound was the largest radioactive component (approximately 22% of the dose in males and 38% in females).

The metabolism was qualitatively similar in male and female rats. The differences occurred however quantitatively. Metabolism of the parent compound to the different metabolites was significantly higher in males compared to female rats.

Based on the results obtained the depletion of radioactive residues from the organs and tissues of male and female rats and metabolism of [furanone-4-^{14}C]BYI 02960 are assumed as adequately understood.

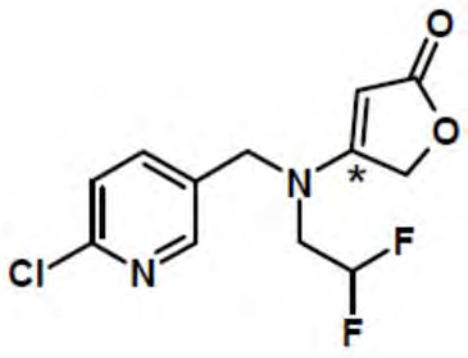
The metabolism results are in good accordance with those obtained from the ADME rat study which was conducted with the [furanone-4-^{14}C] radiolabelled test compound (Weber, 2011b; Study No M1824560-6).

This study is fully reliable (acceptable/guideline) and satisfies the study requirements of a metabolism study (U.S. EPA 870.7485; OECD Section 4 Part 417 and Annex to Directive 87/302/EEC, Part B, Toxicokinetic; JMAFF 12 Nousan 8147).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI02960
   Batch #'s: BYI 02960-PU-02 (exp 27 Oct 2010)
   Purity: 99.4%
   Description: White powder
   CAS #: 951659-40-8
   Stability of test compound: Stated to be stable for two years if stored at 4°C (assay date 27 Oct 2008).
2. Radiolabeled test material: [furanone-4-^{14}C]BYI02960
   Lot/Batch #: KML 9029
   Chemical structure /positions of radiolabel:



* Denotes position of radiolabel

Radio-chemical purity:	[Furanone-4-^{14}C]BYI02960: >99% (HPLC), >99% (TLC)
Specific activity:	[Furanone-4-^{14}C]BYI02960: 106.46 µCi/mg
Description:	Oil, dried in vacuo
Stability of test compound:	The positions of the radiolabels are considered to be stable.

3. Vehicle and/or positive control: 0.5% aqueous Tragacanth®

4. Test animals

Species:	Rat
Strain:	Wistar Hsd/Cpb: WU
Age at dosing:	6 to 9 weeks (males), 10 to 13 weeks (females)
Weight at dosing:	205 to 213 g (males), 201 to 206 g (females)
Source:	Harlan Netherlands BV, Kreuzelweg 53, NL-5960 AD Horst, Netherlands.
Acclimation period:	Seven days in Makrolon® metabolism cages prior to dosing.
Diet:	Rat/mice maintenance long life diet No. 3883.0.15, (Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland), *ad libitum*, except for approximately 16 hours prior to, and for 6 hours after dosing.
Water:	Tap water, *ad libitum*
Housing:	Individually in Makrolon® metabolism cages with wood floor shavings until termination. Cages permitted an 'almost' quantitative separate urine and faeces collection.

5. Environmental conditions

Temperature:	22 to 24°C
Humidity:	47 to 71%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

6. Preparation of the dosing solutions

Rats were dosed with [Furanone-4-^{14}C]BYI02960. The [Furanone-4-^{14}C]BYI02960 radiolabel was diluted with technical BYI02960. The dose vehicle 0.5% aqueous Tragacanth® was added using an ultrasonic bath and stored at 4 - 5°C until use and kept homogenous by magnetic stirring bars and glass balls. Dose level, volume, and radiolabel data are presented in Table 1. The test compound was administered by a single oral gavage dose.

Table 1. Dose Levels, Volumes, and Radiochemical Data

Test No.	**Administration suspension**		
	Total prepared	**Administered per rat**	

	Amount (mg)	Volume (mL)	Volume/ Amount	Target dose (mg/kg bw)	Radioactivity (dpm)	Amount (mg)	Actual dose (mg/kg bw)	Mean rat weight (g)
1	3.12	10.4	2.0 mL/ 0.6 mg	3	141, 613, 690	0.60	2.87	209
2	3.12	10.4	2.0 mL/ 0.6 mg	3	146, 023, 603	0.62	3.03	204

Data excerpted from page 20 of the study report.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

   6-October-2010 to 12-September-2011

2. Main study

   The dose level was based on the specific radioactivity, stability of the dosing suspensions, and the fact that 2 to 5 mg/kg bw doses used as the low dose levels for ADME studies in rats by Weber (2011a, Study no's: M1824567-3; M1824560-6), Klempner (2011; Study no M1824559-4), and the whole body radiography studies of Koester (2011b, M1821760-5) and Koester (2011a, Study no. M1821837-0), had not produced any observable toxicity. The dose level of 3 mg/kg bw was also stated to be consistent with specifications in OECD, US EPA and Japanese MAFF test guidelines on toxicokinetic studies.

   Animals were assigned to test groups according to Table 2 and kept individually in Makrolon® metabolism cages.

Table 2. Study design

Test Group [Sex]	Sampling intervals or time-point (hours)			
	Urine	Faeces	Plasma	Carcass & organs
1 [M]	0 - 6	0 - 6	6	6
2 [F]				

Note: Time of sacrifice was that of time of carcass & organ collection.

3. Plasma Kinetics Following 3 mg/kg bw Oral Dosing

   At six hours post-dosing, animals in both groups were sacrificed by transection of the cervical vessels and exsanguination under Pentobarbital-Na anaesthesia. Oozed out blood was collected in heparinised test tubes and separated afterwards into plasma and blood cells

by centrifugation. After weighing, aliquots from the whole plasma sample were taken for determination of total radioactivity by liquid scintillation counting (LSC).

4. Tissue Distribution at 6 hour sacrifice (3 mg/kg bw)

Tissues and carcass were analyzed to determine ^{14}C tissue distribution by combustion/LSC. In order to get sufficient sample material for extraction of radioactive residues and metabolic profiling, the total radioactivity values of the individual organs and tissue samples were not determined. Instead, respective sample pools were generated for each test group (i.e. sacrifice time per sex). The following tissues were collected at sacrifice from test animals:

Blood (plasma & RBC)	Liver	Kidney
Leg muscle	G.I.T and contents	Skin
Perirenal fat	Carcass	

5. Quantitation of Radioactive Residues

Radioactive residues were measured in plasma, urine (including cage rinse i.e. funnel rinse from the Makrolon® metabolism cage), and tissues. The radioactivity of faeces samples was not determined, because they were added at sacrifice to the gastrointestinal tract (GIT). The measurement of the radioactivity in liquid samples was carried out by LSC. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

6. Quantification and Identification of Metabolites

For metabolic profiling, the urine, plasma, liver, kidney, muscle (leg), and fat (perirenal) samples were pooled across individual animals for a given collection interval for each sex. Liquid chromatography (LC)/mass spectrometry (MS) methods were employed for identification and quantification of the metabolites in each sample. Parent compounds and metabolites were identified by:

- Spectroscopic investigations (LC-MS) of the 0 – 24 h urine from males (test 3) and females (test 6): BYI 02960-difluoroethyl-amino-furanone, isomers 1 and 3 of BYI 02960-OH-gluA, BYI 02960-OH and BYI 02960. Spectroscopic investigation (LC-MS) of the 6 hour plasma from males (test 2) and females (test 5): BYI 02960-DFA, BYI 02960-difluroethyl-amino-furanone, BYI 02960-OH and BYI 02960.
- HPLC comparison of the 0 – 6 h urine sample of males (test 2) and females (test 5) with the radiolabelled reference compounds BYI 02960-difluoroethyl-amino-furanone, and the isomers 1 and 3 of BYI 02960-OH-gluA.
- TLC-co-chromatography of urine and plasma, and extracts from liver, kidney and muscle of males (test 3) and females (test 6) with the radiolabelled reference compound BYI 02960-DFA.

- Comparison of the HPLC-radiochromatograms from all samples of each individual test.
- Comparison of the HPLC-radiochromatograms from the specific samples of male and females: urine, plasma, and extracts from liver, kidney, muscle and fat.

Other peaks or peak groups additionally detected in the HPLC-profiles were designated as "unknown". They were characterised by their behaviour during extraction and clean-up and the retention times in the HPLC-chromatograms.
Parent compound and metabolites were quantified in the original urine and plasma samples and in the conventional acetonitrile/water extracts of organs and tissues by integration of the ^{14}C-signals in the HPLC-chromatograms.

II. RESULTS AND DISCUSSION

A. STORAGE STABILITY

No stability data was reported for the test substance and metabolites in urine stored at <-18°C, nor in freeze dried samples including faeces/GIT contents, liver, kidney, muscle and fat stored at 4°C. Plasma samples were determined to be stable following 138 days storage at <-18°C.

B. MAIN STUDY

Clinical Observation

Clinical signs were not discussed in the study report.

Recovery
The mean total radiolabel recovery from males and females accounted for 100.60% and 98.09% of the administered dose respectively (Table 3).

Table 3: Balance of radioactivity in urine and in organs and tissues of male and female rats sacrificed 6 h after single oral dosing.

Percent of total dose administered (mean arith.)								
Test number	**1**				**2**			
Sex	**M**	**M**	**M**	**M**	**F**	**F**	**F**	**F**
Test period (h)	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Excretion								
Urine	38.29	40.45	36.56	31.27	39.09	39.31	48.56	44.31
Samples								
Plasma	0.78	0.59	0.54	0.88	0.71	0.49	0.53	0.46
Carcass	25.03	24.99	21.66	24.32	25.68	28.59	26.58	26.08
Kidneys	0.74	0.76	0.72	0.71	1.04	0.98	1.02	1.13
Liver	3.42	3.46	3.70	3.66	3.50	3.43	3.42	4.09
GIT+faeces	20.17	22.11	25.77	26.48	11.54	12.72	12.53	16.50
Skin	9.52	8.44	9.04	9.71	10.50	8.96	8.56	9.67
Fat perirenal	0.05	0.05	0.09	0.05	0.09	0.12	0.07	0.07
Leg muscle	1.31	1.00	2.91	3.16	1.00	1.44	1.62	1.01
Balance	**99.32**	**101.90**	**101.00**	**100.20**	**93.14**	**96.03**	**102.90**	**100.30**
Body excluding GIT	40.85	39.30	38.66	42.49	1.07	1.04	0.97	1.00

GIT + faeces	20.17	22.11	25.77	26.48	42.51	44.00	41.80	42.50
Total Body	61.02	61.40	54.42	68.97	54.05	56.72	54.33	56.00

Data excerpted from pages 151 and 153 of the study report.

Absorption

The study authors did not estimate a final absorbed dose. Excluding the gastro-intestinal tract and its contents, the mean demonstrably absorbed fraction of oral dosing by six hours post-dosing was approximately 77% (100.6% minus 23.63%) and 85.52% (98.09% minus 12.57%) for males and females, respectively (Table 3).

Distribution

For both sexes at the 6 hour sacrifice time, approximately 40% of the administered dose was detected in organs and tissues and within this group the highest mean residue values were in the residual carcass (approx. 24 – 27%), followed by skin (approx. 9%), liver (approx. 4%), leg muscle (approx. 1 -2%), kidney (approx. 1%), and perirenal fat which was the lowest value measures (approx. 0.06 – 0.09%) (Table 3).

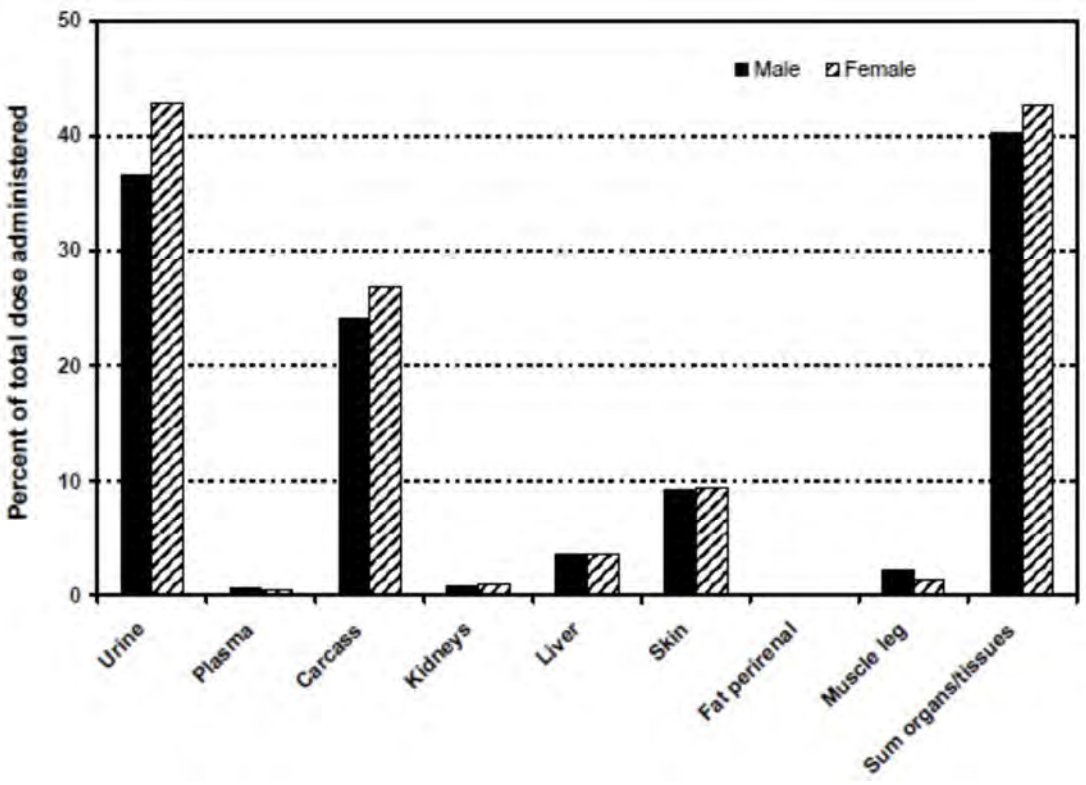


Figure 1: Total radioactivity in plasma, organs and tissues of male and females sacrificed 6 h after single oral dosing.

Figure excerpted from page 73 of the study report.

Excretion

Over the six hour duration of the study, urinary excretion of radiolabel was 36.64% of administered dose in males and 42.82% in females. These results were in good accordance to the urinary excretion behaviour of all other rat studies (Klempner 2011, Study No M1824559-4: Koester 2011b, Study No's M1821760-5 and M1821837-0; Weber 2011a/b, Study No's M1824567-3 and M1824560-6).

Metabolism

Urine

In males and females, the major metabolites of the 0 – 6 hours sample with more was identified as BYI 02960-OH present at 6.9% in males and 3.3% in females. The values for all other metabolites were less than 2% of the dose, and included identified metabolites of BYI2960-difluoroethyl-amino-furanone (1.5% in males, 0.2% in females), BYI2960=des-diflouroethyl (0.8% in both sexes), OH-gluA (isomer 1, 0.4% males) and BYI2960-OH-gluA (isomer 3, 0.9% males). The by far largest component was identified as parent compound (approx. 22.1 and 37.6% of the dose in males and females respectively).

Identification rates of parent compound and metabolites in the 0 – 6 hours urine sample was 88.8% in males and 98.1% in females of the total radioactive residue (TRR).

Plasma

In the plasma, parent compound was the largest component and comprised of 1.087/1.385(M/F) mg/kg sample of total plasma radioactivity. Three minor metabolites - were identified which comprised of BYI2960-difluoroethyl-amino-furanone (0.101/0.012 [M/F] mg/kg), BYI2960-OH (0.068/0.026 [M/F] mg/kg) and BYI2960des-difluoroethyl (0.008 [F] mg/kg). Additionally, unknown metabolites with Peak No 1 (0.056/0.007 [M/F] mg/kg) and 2 (0.010 [F] mg/kg) were detected (Table 4).

Identification rates of parent compound and metabolites in plasma were 95.7% of the TRR in male rats and 98.8% for female rats.

Table 4. Radioactive residue of parent compound and metabolites in the plasma of male and female rats

Test No.		**1**	**2**
Sex		**M**	**F**
Sacrifice after administration		**6 h**	**6 h**
Sampling time		**6 h**	**6 h**
		Equiv. concentration (mg/kg sample)	
Total radioactive residue in plasma pool sample		**1.313**	**1.385**
Compound	*Peak No.*		
Parent	21	1.087	1.385

unknown	1	0.056	0.007
unknown	2	-	0.010
difluoroethyl-amino-furanone	6	0.101	0.012
des-diflouroethyl	13	-	0.008
BYI02960-OH	20	0.068	0.026
Total identified		**1.257**	**1.369**
Total characterised*		0.056	0.016
Sum total		**1.313**	**1.385**

*Peaks were characterised based on their retention time in HPLC-analysis: none of them accounted for >0.056 mg/kg.

Data excerpted from page 52 of the study report.

Liver

In the liver, parent compound was the largest component and comprised of 2.110/2.778 (M/F) mg/kg sample of total liver radioactivity. Identified metabolites were BYI2960-difloroethyl-amino-furanone (0.108/0.015 [M/F] mg/kg), BYI2960-OH-glu A (isomer 1, 0.026 [M] mg/kg, isomer 3 0.055/0.013 [M/F] mg/kg), BYI2960-des-diflouroethyl (0.036/0.024 [M/F] mg/kg) and BYI2960-OH (0.242/0.077 [M/F] mg/kg). Additionally in males only, unknown metabolites with Peak No 1 (0.150 mg/kg), No 2 (0.015 mg/kg), No 14 (0.012 mg/kg), No 17 (0.007 mg/kg) and No 18 (0.016 mg/kg) were detected (Table 5).

Identification rates of parent compound and metabolites in liver were 94.9% of the TRR in male rats and 99.0% for female rats.

Table 5. Radioactive residue of parent compound and metabolites in the liver of male and female rats

Test No.		**1**	**2**
Sex		**M**	**F**
Sacrifice after administration		**6 h**	**6 h**
Sampling time		**6 h**	**6 h**
		Equiv. concentration (mg/kg sample)	
Total radioactive residue in plasma pool sample		**2.928**	**2.937**
Compound	*Peak No.*		
Parent	21	2.110	2.778
unknown	1	0.150	-
unknown	2	0.015	-
difluoroethyl-amino-furanone	6	0.108	0.015
OH-gluA (isomer 1)	9	0.026	-
OH-gluA (isomer 3)	12	0.055	0.013
des-diflouroethyl	13	0.036	0.024
unknown	14	0.012	-
unknown	17	0.007	-
unknown	18	0.016	-
BYI02960-OH	20	0.242	0.077
Total identified		**2.577**	**2.908**
Total characterised*		0.200	-
Total identified or characterised		2.778	2.908

Solids (unextractable)	0.144	0.027
Not analysed samples	0.007	0.002
Sum total**	**2.928**	**2.937**

*Peaks were characterised based on their retention time in HPLC-analysis: none of them accounted for >0.150 mg/kg
** Sum total = Total identified or characterised + solids (unextractable) + not analysed samples

Data excerpted from page 54 of the study report.

Kidney

In the kidney, parent compound was the largest component and comprised of 1.969/4.025 (M/F) mg/kg sample of total kidney radioactivity. Identified metabolites were BYI2960-difloroethyl-amino-furanone (0.125 [M] mg/kg), BYI2960-OH-glu A (isomer 1, 0.010 [M] mg/kg, isomer 3 0.020 [M] mg/kg), BYI2960-des-diflouroethyl (0.036/0.047 [M/F] mg/kg) and BYI2960-OH (0.319/0.197 [M/F] mg/kg). Additionally, unknown metabolites with Peak No 1 (0.174/0.060 [M/F] mg/kg) and No 2 (0.017 [M] mg/kg) were detected (Table 6).

Identification rates of parent compound and metabolites in kidney were 90.7% of the TRR in male rats and 98.2% for female rats.

Table 6. Radioactive residue of parent compound and metabolites in the kidney of male and female rats

Test No.		**1**	**2**
Sex		**M**	**F**
Sacrifice after administration		**6 h**	**6 h**
Sampling time		**6 h**	**6 h**
		Equiv. concentration (mg/kg sample)	
Total radioactive residue in plasma pool sample		**2.732**	**4.346**
Compound	*Peak No.*		
Parent	21	1.969	4.025
unknown	1	0.174	0.060
unknown	2	0.017	-
difluoroethyl-amino-furanone	6	0.125	-
OH-gluA (isomer 1)	9	0.010	-
OH-gluA (isomer 3)	12	0.020	-
des-diflouroethyl	13	0.036	0.047
BYI02960-OH	20	0.319	0.197
Total identified		**2.479**	**4.269**
Total characterised*		0.191	0.060
Total identified or characterised		2.671	4.329
Solids (unextractable)		0.059	0.014
Not analysed samples		0.002	0.003
Sum total**		**2.732**	**4.346**

*Peaks were characterised based on their retention time in HPLC-analysis: none of them accounted for >0.174 mg/kg
** Sum total = Total identified or characterised + solids (unextractable) + not analysed samples

Data excerpted from page 56 of the study report.

Muscle (leg)

In the (leg) muscle, parent compound was the largest component and comprised of 1.160/1.435 (M/F) mg/kg sample of total (leg) muscle radioactivity. Identified metabolites were BYI2960-difloroethyl-amino-furanone (0.090/0.009 [M/F] mg/kg), BYI2960-des-diflouroethyl (0.013/0.012 [M/F] mg/kg) and BYI2960-OH (0.083/0.032 [M/F] mg/kg). Additionally, in males only, an unknown metabolite with Peak No 1 (0.026 [M] mg/kg) was detected (Table 7).

Identification rates of parent compound and metabolites in (leg) muscle were 97.4% of the TRR in male rats and 99.9% for female rats.

Table 7. Radioactive residue of parent compound and metabolites in (leg) muscle of male and female rats

Test No.		**1**	**2**
Sex		**M**	**F**
Sacrifice after administration		**6 h**	**6 h**
Sampling time		**6 h**	**6 h**
		Equiv. concentration (mg/kg sample)	
Total radioactive residue in plasma pool sample		**1.382**	**1.489**
Compound	*Peak No.*		
Parent	21	1.160	1.435
unknown	1	0.026	-
difluoroethyl-amino-furanone	6	0.090	0.009
des-diflouroethyl	13	0.013	0.012
BYI02960-OH	20	0.083	0.032
Total identified		**1.346**	**1.487**
Total characterised*		0.026	-
Total identified or characterised		1.372	1.487
Solids (unextractable)		0.010	0.002
Not analysed samples		n.q.	n.q.
Sum total**		**1.382**	**1.489**

*Peaks were characterised based on their retention time in HPLC-analysis: it accounted for 0.026 mg/kg
** Sum total = Total identified or characterised + solids (unextractable) + not analysed samples

Data excerpted from page 58 of the study report.

Perirenal fat

In the (leg) muscle, parent compound was the largest component in males and the only compound identified in females. Parent compound comprised of 0.474/0.650 (M/F) mg/kg sample of total perirenal fat radioactivity. Metabolites were detected in males only, BYI2960-difloroethyl-amino-furanone (0.023 [M] mg/kg) and BYI2960-OH (0.040 [M] mg/kg) (Table 8).

Identification rates of parent compound and metabolites in (leg) muscle were 96.2% of the TRR in male rats and 99.9% for female rats.

Table 8. Radioactive residue of parent compound and metabolites in perirenal fat of male and female rats

Test No.		**1**	**2**
Sex		**M**	**F**
Sacrifice after administration		**6 h**	**6 h**
Sampling time		**6 h**	**6 h**
		Equiv. concentration (mg/kg sample)	
Total radioactive residue in plasma pool sample		**0.558**	**0.651**
Compound	*Peak No.*		
Parent	21	0.474	0.650
difluoroethyl-amino-furanone	6	0.023	-
BYI02960-OH	20	0.040	-
Total identified		**0.537**	**0.650**
Total characterised*		-	-
Total identified or characterised		0.537	0.650
Solids (unextractable)		0.016	<0.001
Not analysed samples		0.005	-
Sum total**		**0.558**	**0.651**

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

Data excerpted from page 60 of the study report.

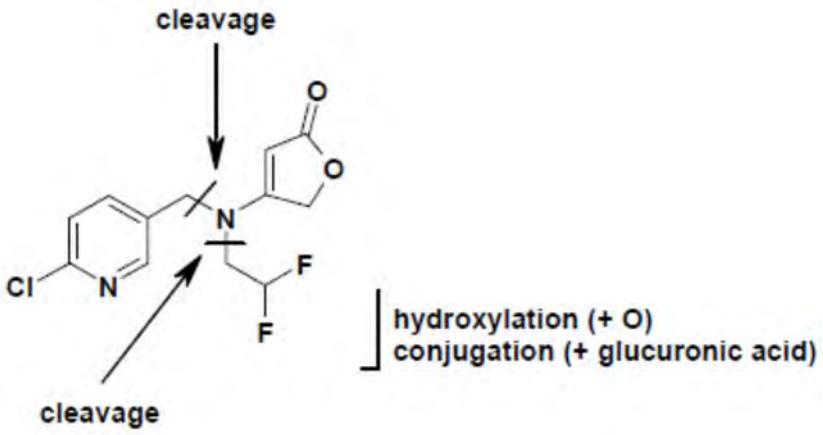
Overall, BYI02960 was metabolised to a comparatively minor extent. Metabolic reactions took place at least at three different structural positions of the test compound. The majority of components were identified (approximately 89 – 100% of TRR in plasma, and in extracts of organs and tissues as well as ≥ 88% of radioactivity in urine samples). In all samples of plasma, and organ tissues the parent compound BYI02960 was by far the largest component accounting for more than 72% of the TRR. For all identified metabolites, the values were less than 12% of the TRR. In the respective urine sample pools, the parent compound was the largest radioactive component (approximately 22% of the dose in males and 36% in females).

The metabolism was qualitatively similar in male and females. The differences occurred however quantitatively. The degradation of the parent compound to the different metabolites was significantly higher in males compared to females.

The principal metabolic reactions of [furanone-4-^{14}C]BYI02960 in the rat were:

- hydroxylation followed by conjugation with glucuronic acid,
- cleavage of the difluoroethyl group forming BYI02960-des-difluoroethyl and
- cleavage of the molecule at the pyridinylmethyl bridge forming BYI 02960-difluoroethyl-amino-furanone.

The following figure shows schematically the positions in the molecule, which are involved in the metabolic reactions:



The proposed biotransformation pathway of [furanone-4-^{14}C]BYI02960 is presented in Figure 1 below.

Figure 1. Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960

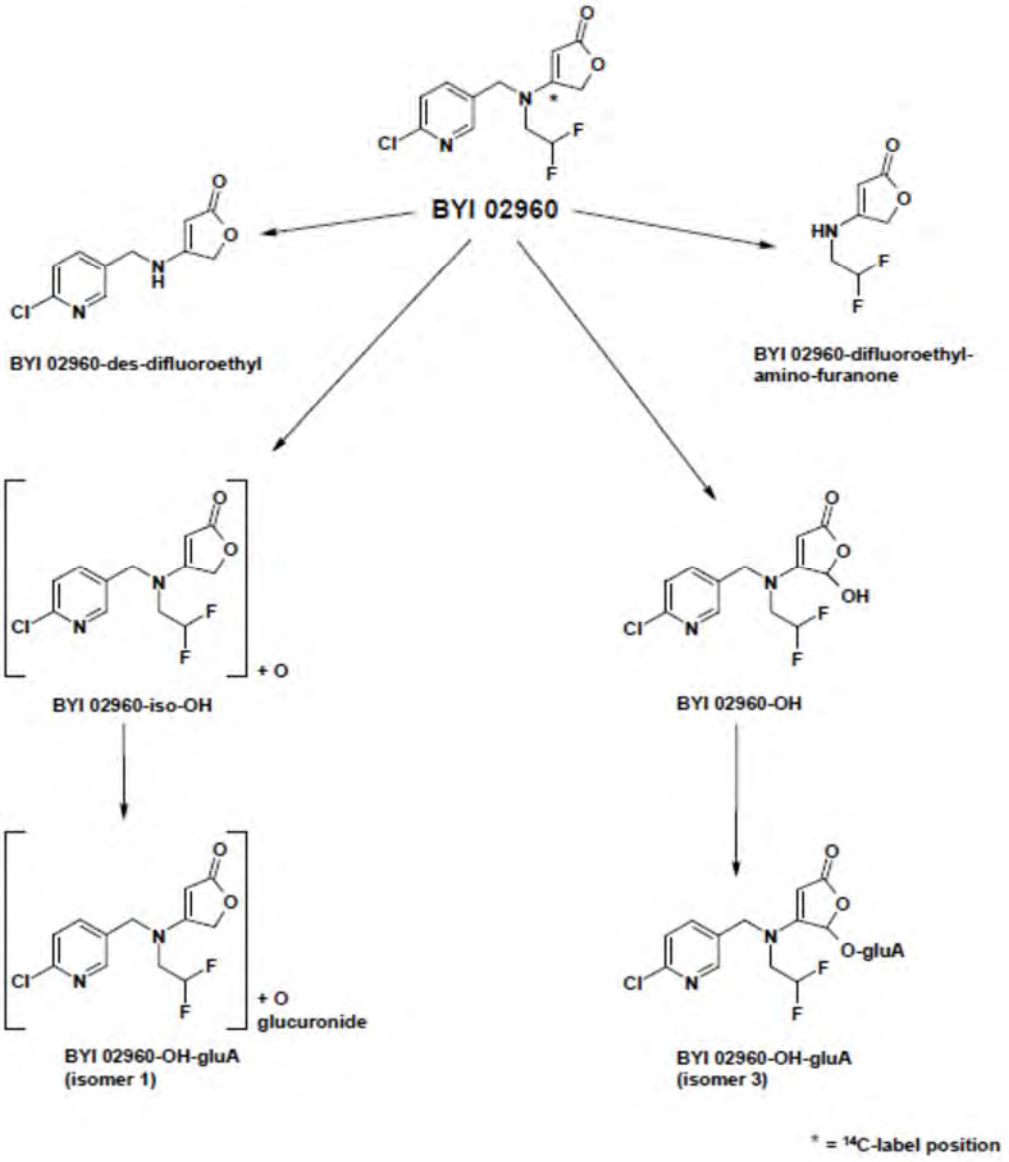


Figure excerpted from page 15 of the study report.

The metabolism results with [furanone-4-^{14}C]BYI02960 are in good accordance with those obtained from the ADME rat study by Weber (2011b; Study M1824560-6).

III. CONCLUSION

The metabolic behaviour of [furanone-4-^{14}C]BYI02960 in Wistar rats can be characterised by the following observations:

- The distribution of the radioactivity within the central compartments (i.e. blood, liver, kidney, muscle and fat) showed a distinctive preference towards the liver and kidney as the main organs responsible for metabolism and excretion.
- Parent compound, major and several minor metabolites were identified in all samples. Identification rates were high with > 88% of radioactivity in urine and > 88%% of TRR in plasma, and organs and tissues.
- The metabolic transformation of BYI 02960 was principally oxidative and took place at least at 3 different structural positions.
- The metabolic pattern was in good accordance with that obtained from the corresponding ADME rat study by Weber (2011b; Study No M1824560-6).
- A clear sex difference was observed for the extent of metabolism, being higher in male than in female rats. The unchanged parent compound was more pronounced in females than in males.

Many examples of sex differences of metabolism in rats have been reported. A very important and general observation is the approximately threefold difference in the activity of hepatic microsomal monooxygenase (cytochrome P-450) in male rats compared to female rats.

Overall, based on the results obtained the depletion of radioactive residues from the organs and tissues of male and female rats and metabolism of [furanone-4-14C]BYI 02960 are assumed as adequately understood.

Primary review: Australia
Secondary review: US EPA

IIA 5.1.1/02 ADME Study in rats: [Ethyl-1-^{14}C]BYI02960

Report: Weber, E (2011a) [Ethyl-1-^{14}C]BYI02960 – Absorption, Distribution, Excretion, and Metabolism in Males. Bayer CropScience AG, Development - Environmental Safety, Metabolism/ADME and Environmental Fate, D-40789 Monheim am Rhein, Germany. Study No. M1824567-3. October. MRID 48844146. Unpublished.

Guidelines: US EPA 870.7485 (1998)
OECD TG 417 (2010)
JMAFF 12 Nousan 8147 (2000)
EC No 1107/2009

Deviations: None

GLP: Yes. Signed and dated GLP, Quality Assurance and Data Confidentiality statements were presented in the report.

Executive summary:

In a toxicokinetic and metabolism study (MRID 48844146**)**, a group of 4 male Wistar rats were administered by oral gavage a single dose of 2 mg/kg bw [Ethyl-1-^{14}C]BYI02960 in 0.5% aqueous Tragacanth® and sacrificed by exsanguination under anaesthesia at 72 hours post dosing. The test compound was radiolabelled with ^{14}C in the C1-position of the ethyl side chain of the molecule. The metabolism and radioactivity which included the test compound and metabolites was determined in urine and faecal samples collected for the time range between dosing and sacrifice. Additionally, radioactivity in plasma micro samples was determined over the study period (23 time points). Total radioactivity was determined in excreta (including expired air) and in organs and tissues collected at sacrifice. Samples were analysed, and parent compound and metabolites identified, by radiochromatographic (HPLC, TLC) and spectroscopic (LCMS) methods.

Mean total radioactivity recovery was nearly quantitative since approximately 100% of the administered dose was found in the excreta and bodies of males at sacrifice.

[Ethyl-1-^{14}C]BYI02960 was nearly completely absorbed since >85% of the total dose administered was detected in the urine and the body without GIT at sacrifice. The absorption started immediately after dosing as could be seen from the quick increase of radioactivity in plasma micro samples, with the peak plasma concentration (C_{max}) was reached approximately 1 hour after administration.

The distribution of the radioactivity within the body was fast. From the maximum plasma level (C_{max}), the radioactivity level declined slowly down to approximately 50% of C_{max} within 8 hours and to approximately 8% of C_{max} 72h after dosing.

Excretion was rapid and mainly by the renal route. Approximately 82% of the total administered dose was excreted in the urine and approximately 14% in the faeces. Only a negligible part of 0.2% of the total administered dose was detected in expired air. The major part of the dose

(>87%) was excreted within 24 hours after treatment, though excretion was ongoing in the time range between 24 and 72 hours the time of sacrifice. In particular, the major part of the metabolite BYI 02960-DFA was excreted on day 2 and day 3. At the time of sacrifice, 72 hours after administration, a small proportion of approximately 3% of the total dose administered was still detected in the body without GIT. The residue was highest in plasma with 0.158 mg/kg sample. For most other organs and tissues, levels were in the range between 0.05 and 0.1 mg/kg sample.

Parent compound, one major (BYI 02960-OH) and five minor metabolites were identified by HPLC and/or TLC co-chromatography or comparison. The label specific metabolite BYI 02960-DFA was additionally identified by high resolution LC/MS of the isolated compound. Identification rates were >95% of the total radioactivity in urine and >85% of the total radioactivity in faeces. Parent compound represented the predominant part of the radioactivity in urine but in faeces samples the metabolite BYI 02960-OH was more prominent. Approximately 92% of the total dose administered was identified in excreta. Another approximate 3% of the dose corresponding to 6 unknown metabolites were characterised by their chromatographic behaviour. All metabolites representing >1% of the dose administered have been identified.

The principal metabolic reactions of [ethyl-1-14C]BYI 02960 in rats were: (1) hydroxylation followed by conjugation with glucuronic acid; (2) cleavage of the difluoroethyl group leading to BYI 02960-DFA; and (3) cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

The results of the metabolism investigations are in good accordance with those obtained from the corresponding organ metabolism rat study with [ethyl-1-14C]BYI 02960 (Koester, 2012a).

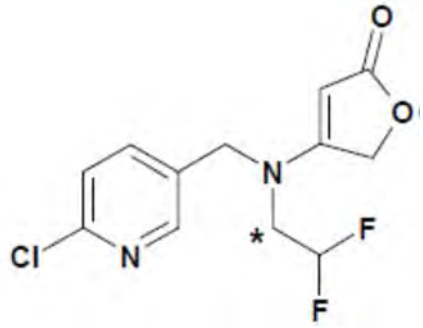
This study is fully reliable (acceptable/guideline) and satisfies the study requirements of a metabolism study (U.S. EPA 870.7485; OECD Section 4 Part 417 and Annex to Directive 87/302/EEC, Part B, Toxicokinetic; JMAFF 12 Nousan 8147).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI02960
   Lot/Batch #:: KATH 6412
   Purity: >99% (HPLC)
   Description: Solid, dried in vacuo
   CAS #: 951659-40-8
   Stability of test compound: Not reported.

Chemical structure /positions of radiolabel:



* Denotes position of radiolabel

Radio-chemical purity:	[ethyl-4-^{14}C]BYI 02960 >99% (HPLC), >99% (TLC)
Specific activity:	[ethyl-4-^{14}C] 106.28 µCi/mg
Description:	Solid, dried in vacuum
Stability of test compound:	The positions of the radiolabels are considered to be stable.

3. Vehicle and/or positive control: 0.5% aqueous Tragacanth®
4. Test animals

Species:	Rat
Strain:	Wistar Hsd/Cpb: WU
Age at dosing:	7 weeks (males)
Weight at dosing:	197 to 201 g (males)
Source:	Harlan Netherlands BV, Kreuzelweg 53, NL-5960 AD Horst, Netherlands.
Acclimation period:	Seven days in Makrolon® metabolism cages prior to dosing.
Diet:	Rat/mice maintenance long life diet No. 3883.0.15, (Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland), *ad libitum*, except for 16 hours prior to dosing and eight hours post-dosing.
Water:	Tap water, *ad libitum*.
Housing:	Individually in Makrolon® metabolism cages with wood floor shavings. Cages permitted an 'almost' quantitative separate urine and faeces collection.

5. Environmental conditions

Temperature:	22 to 24°C
Humidity:	43 to 51%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

6. Preparation of the dosing solutions

Rats were dosed with [ethyl-4-^{14}C] BYI02960. The [ethyl-4-^{14}C] BYI02960 radiolabel was diluted with technical BYI02960. The dose vehicle 0.5% aqueous Tragacanth® was added using an ultrasonic bath and stored at 5°C until use and kept homogenous by magnetic stirring bars and glass balls. Dose route, dose level, volume, and radiolabel data are presented in Table 1. The test compound was administered by a single oral gavage dose.

Table 1. Dose Levels, Volumes, and Radiochemical Data

Test No.	Administration suspension							
	Total prepared		Administered per rat					
	Amount (mg)	Volume (mL)	Volume/ Amount	Target dose (mg/kg bw)	Radioactivity (dpm)	Amount (mg)	Actual dose (mg/kg bw)	Mean rat weight (g)
1	2.4	12	2.0 mL /0.4 mg	2	98, 25, 177	0.42	2.09	199

Data excerpted from page 18 of the study report.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

21 January to 12 November 2010

2. Study design

The dose level was based on the specific radioactivity, stability of the dosing suspensions, and the fact that 2 to 5 mg/kg bw doses used as the low dose levels for ADME studies in rats by Klempner (2011, Study no. M1824559-4), Weber (2011b, Study no. M1824560-6), Koester (2012a/b, Study no's M1824575-2; M1824574-1; and M1821760-5), and Koester (2011a, Study no. M1821837-0) had not produced any observable toxicity. The dose level of 2 mg/kg bw was also stated to be consistent with specifications in OECD, US EPA and Japanese MAFF test guidelines on toxicokinetic studies. Animals were assigned to test groups according to Table 2.

Table 2. Study design

Group	No. of animals	Achieved oral dose (mg/kg bw)	Sampling intervals or timepoint (hours)			
			Urine	Faeces	Expired Air	Plasma microsample
1	4 males	2.09	0 – 4, 4 – 8, 8 – 12, 12 – 24, 24 – 48, 48 – 72	0 – 24, 24 – 48, 48 - 72	0 – 24, 24 – 48, 48 - 72	10 min, 20 min, 40 min, 1, 1.5, 2, 4, 8, 24, 32, 48, 72

Note: Time of sacrifice was 72 hours post-dosing.

3. Plasma Kinetics Following Dosing

In-life blood samples from individual animals were obtained by pressing a heparin coated capillary into a small cut in the tail vein which was subsequently closed by tape. Blood samples were separated afterwards into plasma and blood cells by centrifugation. At 72 hours post-dosing animals in each group were sacrificed by transection of the cervical vessels and exsanguination under Pentobarbital-Na anaesthesia. Oozed out blood samples from individual animals was collected in heparinised test tubes and separated afterwards into plasma and blood cells by centrifugation. After weighing, aliquots from the whole plasma sample were taken for determination of total radioactivity by liquid scintillation counting (LSC).

4. Tissue Distribution at Sacrifice

Tissues and carcass were analyzed to determine ^{14}C tissue distribution by combustion/LSC. The following tissues were collected at sacrifice from test animals:

Blood (plasma & RBC)	Liver	Femur
Kidney	Leg muscle	Heart
Lung	Testes	Femur
Brain	Spleen	Adrenals
Thyroid	GIT (inc faeces)	Skin
Harderian gland	Eyes	Perirenal fat

5. Quantitation of Radioactive Residues

Radioactive residues of all individual animals were measured in plasma, urine (including cage rinse i.e. funnel rinse from the Makrolon® metabolism cage) and faeces samples, and tissues. The measurement of the radioactivity in liquid samples was carried out by LSC. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

6. Calculation of toxicokinetic parameters

Standard toxicokinetic parameters for plasma were calculated, such as.

$AUC_{0-\infty}$	Area under the plasma radioactivity concentration-time curve extrapolated from time 0 to infinity
$t_{1/2\ a}$	half-life of the absorption
$t_{1/2\ e}$	half-life of the elimination (terminal half-life)
C_{max}	maximum radioactivity concentration in plasma after single (first) dose administration

t_{max}	time at which the highest radioactivity concentration occurs in plasma after a single dose
MRT	mean residence time describing the sojourn (elimination and/or metabolism) of the chemical (or total radioactivity) in the body

The evaluation was performed with dose normalised values for the plasma concentrations. A standard two compartment disposition model was used for curve fitting computation.

7. Quantification and Identification of Metabolites

For metabolic profiling, the urine and faecal samples were pooled across individual animals for a given collection interval for each sex. Liquid chromatography (LC)/mass spectrometry (MS) methods were employed for identification and quantification of the metabolites in each sample. The identification of metabolites was mainly based on comparison of the urine profile with results of the organ metabolism study with [ethyl-1-14C]BYI 02960 (Koester, 2012a; Study No. M1824575-2). The label specific metabolite BYI 02960-DFA was isolated from urine by repeated HPLC fraction with method BYI02960_FRAK1 and subjected to high resolution LC/MS
analysis. Additional identification for parent and DFA was performed by HPLC and TLC co-chromatography.

In the Koester (2012a) organ metabolism study with [ethyl-1-^{14}C]BYI 02960 (Study No. M1824575-2), parent compounds and metabolites were identified by:

- spectroscopic evidence (LC-MS and high resolution LC-MS analysis, and 1H-NMR and 2D-NMR analysis) following isolation and purification from urine pool,
- HPLC and TLC co-chromatography of urine samples or isolated peaks with the isolated and identified metabolites and with non-radiolabelled reference compounds,
- HPLC co-chromatography with egg extract and the isolated metabolite (BYI02960-OH-SA) from egg extract of the laying hen metabolism study performed with [pyridinylmethyl-14C]BYI 02960,
- enzyme treatment of isomer 1 and isomer 3 of BYI 02960-OH-gluA with glucuronidase / arylsulfatase along with comparison of the HPLC profiles of the metabolites before and after cleavage to the profiles of urine, faeces extract and reference compounds,
- comparison of the HPLC profiles of urine pools of the several time intervals of test 1 among themselves and with the ones of all other tests, and
- comparison of the HPLC profiles of all faeces extracts among themselves and with the profiles of urine.

Other peaks or peak groups additionally detected in the HPLC-profiles were designated as "unknown". They were characterised by their behaviour during extraction and clean-up and the retention times in the HPLC-chromatograms.

II. RESULTS AND DISCUSSION

A. STORAGE STABILITY

No stability data was reported for the test substance and metabolites in plasma and urine stored at <-18°C, nor in freeze dried samples including faeces stored at 4°C.

B. MAIN STUDY

Clinical Observation

There were no reported clinical signs of toxicity.

Recovery
Mean total radioactivity recovery was approximately 100% of the administered dose in males. The majority of radioactivity was recovered from the urine, and together with the moderate recovery from faeces, excreta accounted for almost all of the recovered radioactivity. A minimal amount of radioactivity was recovered from expired air (Table 3).

Table 3: Balance of radioactivity in excreta and in organs and tissues (Percent of total dose administered)

	Animal No.				**Mean**	**CV[%]**
	928	**929**	**930**	**931**		
Expired air 1	0.07	0.05	0.07	0.07	0.07	14.8
Expired air 2	0.15	0.10	0.14	0.15	0.13	17.3
Faeces	15.34	13.82	11.39	13.47	13.51	12.0
Urine	82.21	82.79	77.32	86.64	82.24	4.7
Sum of excreta	97.77	?96.76	88.92	?100.30	95.95	5.1
Body excluding GIT	3.46	2.64	3.35	3.30	3.19	11.6
GIT	0.85	0.62	0.73	0.70	0.73	13.0
Total Body	4.31	3.27	4.08	4.00	3.92	11.6
Balance	**102.10**	**100.00**	**93.00**	**104.30**	**99.86**	**4.9**

Data excerpted from page 94 of the study report.

Absorption

The pharmacokinetic results indicated that absorption occurred immediately following administration of a single (2 mg/kg bw) oral gavage dose. Gastrointestinal absorption of the test substance was at least 85% in males, as >85% of the total dose administered was detected in urines and in the carcass excluding GIT. The mean peak plasma concentration (C_{max}) was reached approximately 1 hour after administration (Table 4).

Table 4: Time course of radioactivity measured in the plasma following a single oral dosing of 2 mg/kg bw [ethyl-1-^{14}C]BYI02960 to males (Equivalent concentration [mg a.s. equiv. /kg sample])

	Animal No.				**Mean**	**CV[%]**
Time (hours)	**928**	**929**	**930**	**931**		
0.17	0.585	0.323	1.329	0.415	0.663	69.0

0.33	1.269	1.035	2.124	1.135	1.391	35.8
0.37	1.945	1.737	2.309	1.520	1.878	17.9
1	2.094	1.964	2.292	1.717	2.017	12.0
1.5	2.021	1.954	2.104	1.770	1.963	7.2
2	1.898	1.943	2.051	1.751	1.911	6.5
4	1.358	1.598	1.731	1.438	1.532	10.9
8	0.897	1.076	1.162	0.847	0.995	14.9
24	0.368	0.309	0.373	0.378	0.357	9.1
32	0.374	0.301	0.384	0.375	0.358	10.8
48	0.255	0.185	n.d.	0.249	0.230	16.9
72	0.182	0.123	0.168	0.176	0.162	16.3

Data excerpted from page 97 of the study report.

The plasma concentrations declined to approximately 50% of C_{max} within 8 hours and to approximately 10% of Cmax within 48 hours. At the time of sacrifice, 72 hours after administration, the plasma concentrations had further reduced to 8% of C_{max} Biokinetic modelling of mean values between 0 to 72 hours was undertaken, using a two compartment model. The corresponding concentration-time curve of the modelled plasma data is shown in Figure 1.

Figure 1: Time course of total radioactive residues in plasma

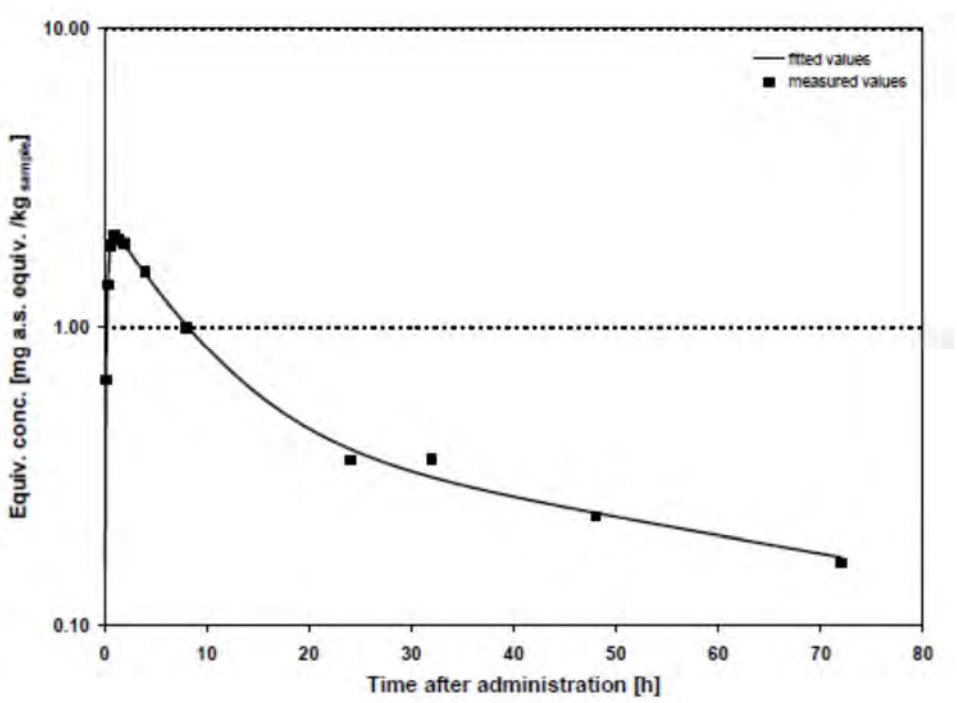


Figure excerpted from page 59 of the study report.

The AUC extrapolated from 0 to infinity amounted to 45.6 mg/L/h, the half-life of absorption and elimination were 0.2 and 50.4 hours respectively, and the mean residence time was 54.8 hours. A summary of the pharmacokinetic pararmeters are shown in Table 5.

Table 5: Pharmacokinetic parameters of [ethyl-1-14C]BYI02960 after oral dosing to males

Parameter	Value
t_{max} [h] measured	1.00
t_{max} [h] modelled	1.06
C_{max} [mg/kg] measured	2.02
C_{max} [mg/kg] modelled	2.03
$t_{1/2abs}$ [h]	0.2
$t_{1/2elim}$ [h]	50.4
$AUC_{0-\infty}$ [mg/kg*h]	45.6
MRT [h]	54.8

Data excerpted from page 49 of the study report.

Distribution

A mean of approximately 4% of the total administered dose was detected in the body at the time of sacrifice 72 hours after oral dosing, of which 0.73% was in the GIT and 3.19% in the body without GIT. Mean residual concentrations in tissues demonstrated a wide distribution, and ranged from 0.025 (Harderian gland) to 0.158 mg/kg sample (plasma). Levels for most organs and tissues were very similar and in the range of approximately 0.05 to 0.1 mg/kg sample (Table 6).

Table 6: Radioactive residues in organs and tissues at sacrifice following a single oral administration of 2 mg/kg bw [ethyl-1-14C]BYI 02960 to male rats (Equivalent concentration [mg a.s. equiv. /kg sample])

Organ/tissue	Animal No.				Mean	CV[%]
	928	929	930	931		
Blood cells	0.121	0.083	0.120	0.090	0.104	19.1
Plasma	0.180	0.121	0.161	0.170	0.158	16.5
Carcass	0.071	0.054	0.066	0.065	0.064	11.0
Heart	0.069	0.068	0.073	0.102	0.078	20.7
Brain	0.076	0.087	0.091	0.077	0.083	8.9
Kidneys	0.075	0.044	0.080	0.065	0.066	23.7
Liver	0.107	0.072	0.091	0.110	0.095	18.1
GIT	0.159	0.094	0.132	0.129	0.129	20.7
Testes	0.074	0.052	0.066	0.069	0.065	14.7
Adrenal gland	0.080	0.056	0.079	0.075	0.073	15.6
Harderian gland	0.027	0.023	0.021	0.029	0.025	15.0
Thyroid gland	0.100	0.065	0.090	0.096	0.088	17.6
Spleen	0.081	0.058	0.078	0.082	0.075	14.9
Lung	0.099	0.068	0.095	0.091	0.088	15.8
Eye	0.138	0.162	0.093	0.160	0.138	23.3
Skin	0.082	0.061	0.087	0.085	0.079	14.9
Bone femur	0.059	0.043	0.054	0.051	0.052	12.8
Fat perirenal	0.059	0.037	0.063	0.056	0.054	21.7
Leg muscle	0.061	0.042	0.059	0.058	0.055	15.6

Data excerpted from page 98 of the study report.

Excretion

Excretion was rapid and almost complete by 72 hours post dosing with a mean of approximately 96% of total administered dose being excreted by this time point. The major route of excretion was urine, accounting for approximately 82% of the dose, with approximately 76% of total dose being excreted by this route within 24 hours. Faecal excretion accounted for approximately 14% of the total administered dose, with approximately 11% being excreted within 24 hours. A very low proportion of the total administered dose was detected in expired air, 0.2% over 72 hours post-dosing with the majority of this fraction being excreted within the first 24 hours (Table 7).

Table 7: Time course of excretion of radioactive residues via urine, faeces, and expired air following a single oral administration of 2 mg/kg bw [ethyl-1-14C]BYI 02960 to male rats (Cumulative excretion in percent of total dose administered)

Excretion time (h)	**Animal No.**				**Mean**	**CV[%]**
	928	**929**	**930**	**931**		
Expired Air (trapped in 10% aqueous ammonia)						
24	0.15	0.12	0.15	0.17	0.14	15.3
48	0.2	0.14	0.19	0.2	0.19	15.3
72	0.22	0.1	0.21	0.15	0.2	17.3
Faeces						
24	11.93	11.62	8.94	11.82	11.08	12.9
48	14.91	13.39	10.97	13.19	13.12	12.4
72	15.34	13.82	11.39	13.47	13.51	12.0
Urine						
4	4.62	16.72	9.69	28.76	14.95	70.0
8	40.10	45.36	41.60	58.53	46.40	18.1
12	60.76	55.74	56.80	68.61	60.48	9.7
24	75.38	75.77	72.18	81.16	76.12	4.9
48	80.41	81.40	75.52	85.08	80.60	4.9
72	82.21	82.79	77.32	86.64	82.24	4.7
Sum total	**97.77**	**96.76**	**88.92**	**100.30**	**95.95**	**5.1**

Data excerpted from page 95 of the study report.

Metabolism

Urine

Over 72 hours, the main compound in urine was parent BYI 02960 representing approximately 52% of the total dose administered to male rats. Only one major metabolite was detected, BYI 02960-OH, which accounted for approximately 16% of the total administered dose. Additionally, BYI 02960-DFA was found at a level of approximately 5% of the total dose administered, and was predominantly excreted in the time range between 24 and 72 hours after administration. Other identified metabolites were BYI 02960-difluoroethyl-aminofuranone (approximately 4%), BYI 02960-OH-gluA isomer 1 (approximately 1%), BYI 02960-OH-gluA isomer 3(approximately 2%) which accounted for less than 4% of the dose each. In total, approximately 82% of the total administered dose was detected in urine of which approximately 80% were

identified and approximately 2% were characterised but not identified. All four characterised metabolites were found at levels of <1% of the total dose administered (Table 8).

Faeces

Over 72 hours, the main compound in faeces was the metabolite The main constituent of the faecal residues was metabolite BYI 02960-OH which accounted for approximately 8% of the total dose administered. The parent compound BYI 02960 was present at approximately 4%. Additionally, BYI 02960-DFA and BYI 02960-iso-OH were found at levels of approximately 0.5% and 0.4% of the total dose administered respectively. In total, approximately 14% of the total administered dose were detected in faeces of which approximately 12% were identified and approximately 0.3% were characterised but not identified. All two characterised metabolites were found at levels of <0.2% of the total dose administered (Table 8).

Table 8: Percent of dose of parent compound and metabolites in urine and faeces

Sample		**Urine**							**Faeces**			
Time [h]		**0-4**	**4-8**	**8-12**	**12-24**	**24-48**	**48-72**	**Total**	**0-24**	**24-48**	**48-72**	**Total**
Peak ID	**Compound (report name) BYI 2960-**											
12	**parent**	11.54	22.46	8.05	8.79	1.11	---	**51.96**	3.64	0.15	---	**3.79**
1	DFA	---	0.36	0.43	1.06	1.78	1.64	**5.28**	0.20	0.29	---	**0.49**
6	difluoroethyl-amino-furanone	0.42	1.50	0.91	0.66	0.14	---	**3.63**	---	---	---	**---**
7	OH-gluA (isomer1)	0.17	0.20	0.29	0.50	0.25	---	**1.40**	---	---	---	
8	OH-gluA (isomer 3)	0.33	0.57	0.30	0.44	0.15	---	**1.79**	---	---	---	
11	OH	2.49	5.51	3.31	3.77	1.05	---	**16.13**	6.26	1.34	---	**7.60**
13	Iso-OH	---	---	---	---	---	---	---	0.27	0.16	---	**0.43**
Total identified		**14.95**	**30.61**	**13.29**	**15.22**	**4.48**	**1.64**	**80.19**	**10.37**	**1.94**	**---**	**12.31**
2	Unknown 1	---	0.14	0.30	0.20	---	---	**0.64**	---	---	---	**---**
3	Unknown 2	---	0.24	0.23	0.22	---	---	**0.68**	---	---	---	**---**
4	Unknown 3	---	0.25	0.11	---	---	---	**0.36**	---	---	---	**---**
5	Unknown 4	---	0.21	0.16	---	---	---	**0.37**	---	---	---	**---**
9	Unknown 5	---	---	---	---	---	---	---	0.10	---	---	**0.10**
10	Unknown 6	---	---	---	---	---	---	---	0.17	---	---	**0.17**
Total characterised		---	0.84	0.79	0.42	---	---	**2.04**	0.27	---	---	**0.27**
Total in extracts		---	---	---	---	---	---	---	**10.64**	**1.94**	**---**	**12.58**
Post extraction solids(PES) of faeces		---	---	---	---	---	---	---	0.39	0.10	---	0.49
Not analysed samples		---	---	---	---	---	---	---	0.04	0.01	0.39	0.44
Total in urine/faeces extracts		**14.95**	**31.45**	**14.08**	**15.64**	**4.48**	**1.64**	**82.23**	**11.08**	**2.04**	**0.39**	**13.51**

Note: Samples not analysed were faeces 0 – 24 h, 24 – 48 h and faeces 48 – 72 hr.

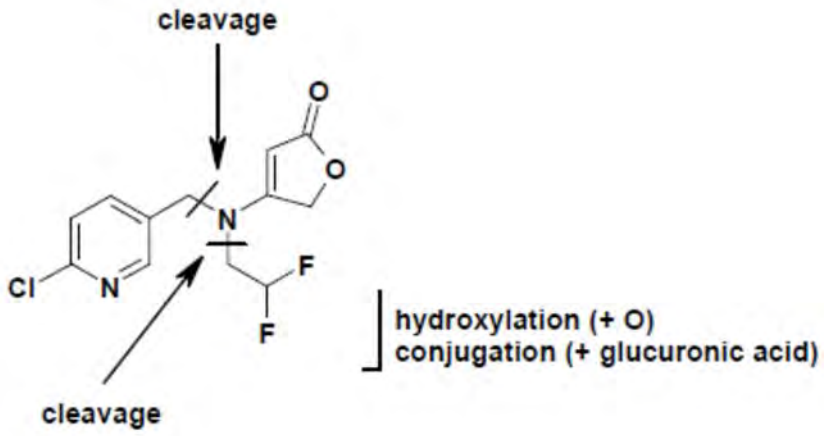
Data excerpted from pages 52 and 53 of the study report.

Overall, BYI 02960 was moderately metabolised to a small number of metabolites. Parent compound represented the predominant part of the radioactivity in urine but in faeces samples the metabolite BYI 02960-OH was more prominent. The metabolite BYI 02960-DFA was both detected in urine and faeces. All other identified and characterised metabolites represented a minor part of the dose and were not common to both urine and faeces.

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

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

The following figure shows schematically the positions in the molecule, which are involved in the metabolic reactions.



The proposed biotransformation pathway of [ethyl-1-^{14}C] BYI02960 is presented in Figure 2.

III. CONCLUSION

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

- The test compound was nearly completely absorbed since >85% of the administered dose was detected in the urine and the body without GIT at sacrifice.
- The distribution of the radioactivity within the body was rapid and the maximum plasma level (Cmax) was reached within one hour after administration. From the maximum, the radioactivity level declined slowly down to approximately 50% of Cmax after 8 hours and approximately 8% of Cmax at the time of sacrifice 72 h after dosing.
- Excretion of BYI 02960 related radioactivity was fast and occurred mainly by the renal route. The major part of the dose (>87%) was excreted within 24 hours after treatment. But excretion was ongoing in the time range between 24 and 72 hours. In particular, the major part of the metabolite BYI 02960-DFA was excreted on day 2 and day 3. Parent compound represented the predominant part of the radioactivity in urine but in faeces samples the metabolite BYI 02960-OH was more prominent. A very low proportion of the total administered dose was detected in expired air, 0.2% over 72 hours post-dosing with the majority of this fraction being excreted within the first 24 hours

- At the time of sacrifice, 72 hours after administration, a small proportion of ca. 3% of the dose administered was still detected in the body without GIT. The residue in plasma was 0.158 mg/kg; for most other organs and tissues levels were in the range between 0.05 and 0.1 mg/kg.
- Parent compound, one major (BYI 02960-OH) and five minor metabolites were identified in all samples. Identification rates were >95% of the total radioactivity in urine and >85% of the total radioactivity in faeces.
- Approx. 92% of the total dose administered was identified in excreta. Another approximate 3% of the dose corresponding to 7 unknown metabolites were characterised by their chromatographic behaviour. All metabolites representing >1% of the dose administered were identified.
- The metabolic transformation of BYI 02960 was principally oxidative and took place at least at 3 different structural positions of the test compound.
- The metabolic pattern was in good accordance with that obtained from the corresponding organ metabolism rat study with [ethyl-1-^{14}C]BYI 02960 (Koester, 2012a; Study No. M1824575-2).

Based on the results obtained the absorption, distribution, excretion, and metabolism of [ethyl-1-14C]BYI 02960 in rats are assumed as adequately understood.

Figure 2. Proposed metabolic pathway of [ethyl-1-^{14}C]BYI 02960 in male rats

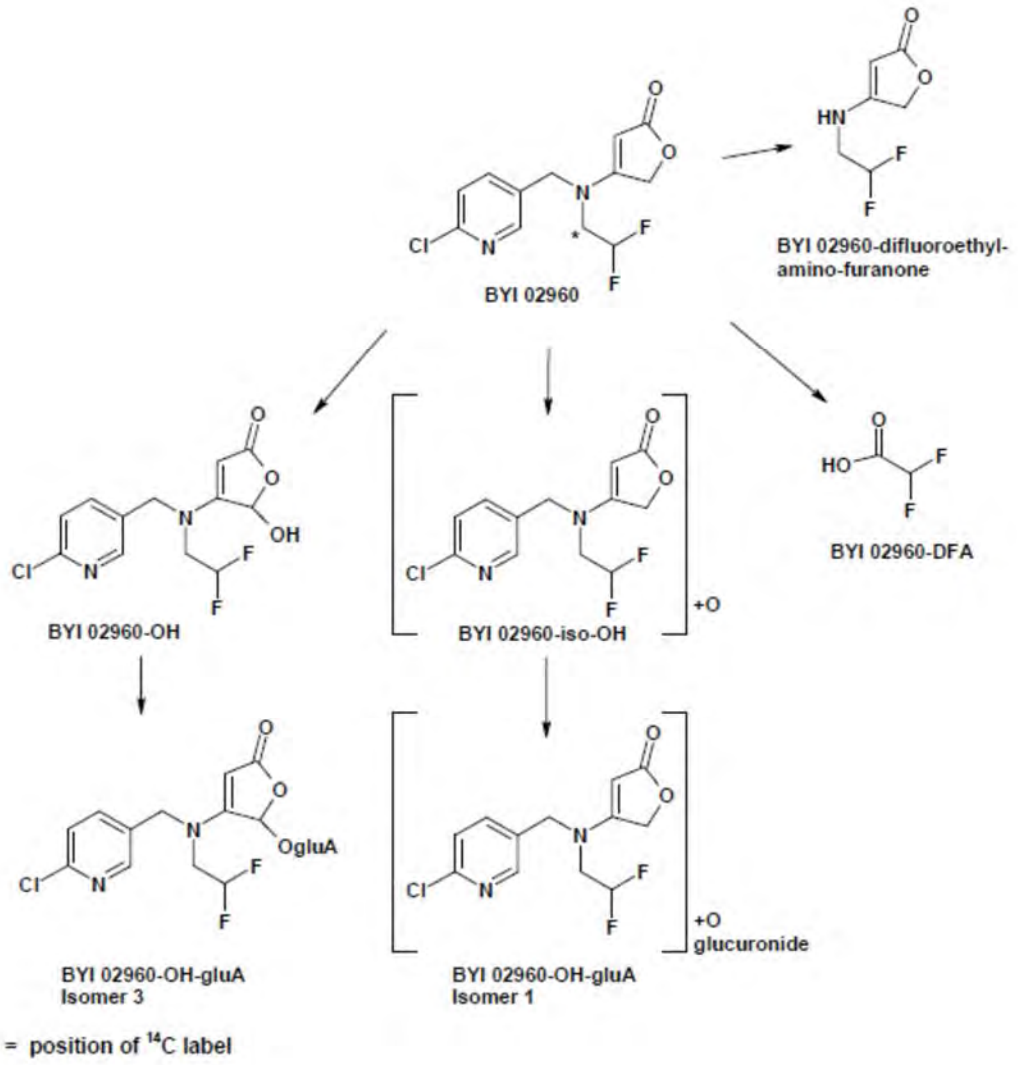


Figure excerpted from page 80 of the study report.

Primary review: Australia
Secondary review: US EPA

IIA 5.1.1/04 Metabolism study in rats: [Ethyl-1-^{14}C]BYI02960

Report: Koester J (2012a) [Ethyl-1-^{14}C]BYI02960 - Metabolism in organs and tissues of male and females (3 time-points). Bayer CropScience AG, Development - Environmental Safety, Metabolism/ADME and Environmental Fate, D-40789 Monheim am Rhein, Germany. Study No. M1824575-2. September. MRID 48844147. Unpublished.

Guidelines: US EPA 870.7485 (1998)
OECD TG 417 (2010)
JMAFF 12 Nousan 8147 (2000)
Deviations: None

GLP: Yes. Signed and dated GLP, Quality Assurance and Data Confidentiality statements were presented in the report.

Executive summary:

In a metabolism study, groups of 4 male and 4 female Wistar rats per sacrifice time were administered by oral gavage a single dose of 3 mg/kg bw [ethyl-1-^{14}C]BYI02960 in 0.5% aqueous Tragacanth® and sacrificed by exsanguination under anaesthesia at 1, 6, or 24 hours post dosing. The test compound was radiolabelled with ^{14}C in the C1-position of the ethyl side chain of the molecule. The metabolism and total radioactivity which included the test compound and metabolites was determined in urine samples for the time periods 0 – 1 h, 0 – 6 h and 0 – 24 h as well as in plasma, liver, kidney, muscle (leg) and fat (perirenal) at sacrifice. Samples were analysed, and parent compound and metabolites identified, by radiochromatographic (HPLC, TLC) and spectroscopic (LCMS) methods.

Mean total radiolabel recovery over the sacrifice times in males ranged from 95.85% to 98.03% of the administered dose, and in females ranged from 100.3 to 103.7%. The urinary excretion started immediately after administration and increased until 24 hours to 71.80% of the administered dose in male and 85.88% in female rats.

The distribution of the radioactivity within the central compartments of the body (e.g. blood, liver, and kidney) was fast. For both sexes, the highest radioactivity residue values in organs and tissues combined were detected at the one hour time-point with approximately 64.6/81.0% (M/F) of the administered dose, declining substantially by 24 hours post dosing to less than 8% (M/F). Radioactivity residues showed a distinctive preference towards the liver and kidney as the main organs responsible for metabolism and urinary excretion. Highest levels at one hour were in kidney (4.7/4.9 mg/kg sample for M/F) and liver (4.3/5.8 mg/kg sample for M/F) followed by leg muscle (2.3/2.9 mg/kg sample for M/F), skin (2.0/2.4 mg/kg sample for M/F), and fat (0.9/1.0 mg/kg sample for M/F). By 24 hours levels in the organs/tissues fell to 4 to 12% of 1 hour levels.

It is expected that the small residual amounts are further eliminated smoothly from the body. Indications of irreversible binding or retention of radioactivity in organs and tissues are therefore not expected.

Metabolism of BYI02960 was extensive. The majority of metabolic components were identified

in both sexes (approximately 97 – 100% of total radioactive residue (TRR) in plasma, and in extracts of organs and tissues as well as ≥ 95% of radioactivity in urine samples). Metabolic reactions took place at least at 3 different structural positions of the test compound. The principal metabolic reactions of [ethyl-1-14C]BYI 02960 in rats were: (1) hydroxylation followed by conjugation with glucuronic acid, (2) cleavage of the difluoroethyl group leading to BYI 02960-DFA and (3) cleavage of the molecule at the pyridinylmethyl bridge leading to BYI 02960-difluoroethyl-amino-furanone.

In the 24 hours samples of plasma, and organs and tissues BYI 02960-DFA was the by far largest metabolite accounting for more than 50% of the TRR. For all other identified metabolites, the values were less than 10% of the TRR. The percentage-values of the parent compound in these samples ranged from 6 to 38% of the TRR. In the respective urine sample pools, the parent compound was the largest radioactive component (approximately 48% of the dose in males and 77% in females).

The metabolism was qualitatively similar in male and female rats. The differences occurred however quantitatively. Metabolism of the parent compound to the different metabolites was significantly higher in males compared to female rats.

Based on the results obtained the depletion of radioactive residues from the organs and tissues of male and female rats and metabolism of [ethyl-1-^{14}C]BYI 02960 are assumed as adequately understood.

The metabolism results are in good accordance with those obtained from the ADME rat study which was conducted with the [ethyl-1-^{14}C] radiolabelled test compound (Weber, 2011a; Study No M1824567-3).

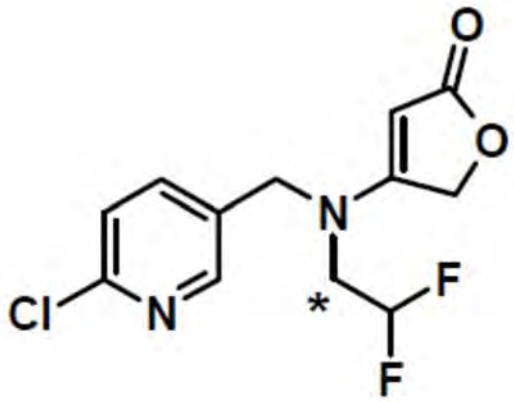
This study is fully reliable (acceptable/guideline) and satisfies the study requirements of a metabolism study (U.S. EPA 870.7485; OECD Section 4 Part 417 and Annex to Directive 87/302/EEC, Part B, Toxicokinetic; JMAFF 12 Nousan 8147).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI02960
   Batch #'s: BYI 02960-PU-02 (exp 27 Oct 2010)
   Purity: 99.4%
   Description: White powder
   CAS #: 951659-40-8
   Stability of test compound: Stated to be stable for two years if stored at 4°C (assay date 27 Oct 2008).
2. Radiolabeled test material: [ethyl-1-^{14}C]BYI02960
   Lot/Batch #: KML 9028

Chemical structure /positions of radiolabel:



* Denotes position of radiolabel

Radio-chemical purity:	[ethyl-1-^{14}C]BYI02960: >99% (HPLC), >98% (TLC)
Specific activity:	[ethyl-1-^{14}C]BYI02960: 106.28 μCi/mg
Description:	Oil, dried in vacuo
Stability of test compound:	The positions of the radiolabels are considered to be stable.

3. Vehicle and/or positive control: 0.5% aqueous Tragacanth®
4. Test animals

Species:	Rat
Strain:	Wistar Hsd/Cpb: WU
Age at dosing:	7 weeks (males), 9 weeks (females)
Weight at dosing:	193 to 209 g (males), 196 to 208 g (females)
Source:	Harlan Netherlands BV, Kreuzelweg 53, NL-5960 AD Horst, Netherlands.
Acclimation period:	Seven days in Makrolon® metabolism cages prior to dosing.
Diet:	Rat/mice maintenance long life diet No. 3883.0.15 (Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland), *ad libitum*, except for approximately 16 hours prior to, and for 6 hours after dosing.
Water:	Tap water, *ad libitum*
Housing:	Individually in Makrolon® metabolism cages with wood floor shavings until termination. Cages permitted an 'almost' quantitative separate urine and faeces collection.

5. Environmental conditions

Temperature:	20 to 25°C
Humidity:	54 to 77%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

6. Preparation of the dosing solutions

Rats were dosed with [ethyl-1-^{14}C]BYI02960. The [ethyl-1-^{14}C]BYI02960 radiolabel was diluted with technical BYI02960. The dose vehicle 0.5% aqueous Tragacanth® was added using an ultrasonic bath and stored at 5°C until use and kept homogenous by magnetic stirring bars and glass balls. Dose level, volume, and radiolabel data are presented in Table 1. The test compound was administered by a single oral gavage dose.

Table 1. Dose Levels, Volumes, and Radiochemical Data

Test No.	**Administration suspension**							
	Total prepared		**Administered per rat**					
	Amount (mg)	**Volume (mL)**	**Volume/ Amount**	**Target dose (mg/kg bw)**	**Radioactivity (dpm)**	**Amount (mg)**	**Actual dose (mg/kg bw)**	**Mean rat weight (g)**
1	9.0	30	2.0 mL/ 0.6 mg	3	145, 577, 035	0.62	3.03	204
2							3.09	200
3							3.18	194
Mean Tests 1 - 3							**3.10**	**199**
1	9.0	30	2.0 mL/ 0.6 mg	3	146, 767, 166	0.62	3.01	207
2							3.10	201
3							3.16	197
Mean Tests 4 - 6							**3.09**	**202**

Table excerpted from page 21 of the study report.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

5-November-2010 to 29-September-2011

2. Main study

The dose level was based on the specific radioactivity, stability of the dosing suspensions, and the fact that 2 to 5 mg/kg bw doses used as the low dose levels for ADME studies in rats by Weber (2011a, Study no's: M1824567-3; M1824560-6), Klempner (2011; Study no M1824559-4), and the whole body radiography studies of Koester (2011b, M1821760-5) and Koester (2011a, Study no. M1821837-0), had not produced any observable toxicity. The dose level of 3 mg/kg bw was also stated to be consistent with specifications in OECD, US EPA and Japanese MAFF test guidelines on toxicokinetic studies.

Animals were assigned to test groups according to Table 2 and kept individually in Makrolon® metabolism cages.

Table 2. Study design

Test Group [Sex]	Sampling intervals or time-point (hours)			
	Urine	Faeces	Plasma	Carcass & organs
1 [M]	0 - 1	0 - 1	1	1
2 [M]	0 - 6	0 - 6	6	6
3 [M]	0 - 24	0 - 24	24	24
4 [F]	0 - 1	0 - 1	1	1
5 [F]	0 - 6	0 - 6	6	6
6 [F]	0 - 24	0 - 24	24	24

Note: Time of sacrifice was that of time of carcass & organ collection.

3. Plasma Kinetics Following 3 mg/kg bw Oral Dosing

At one, six, or 24 hours post-dosing, animals in each group were sacrificed by transection of the cervical vessels and exsanguination under Pentobarbital-Na anaesthesia. Oozed out blood samples from individual animals was collected in heparinised test tubes and separated afterwards into plasma and blood cells by centrifugation. After weighing, aliquots from the whole plasma sample were taken for determination of total radioactivity by liquid scintillation counting (LSC).

4. Tissue Distribution at sacrifice (3 mg/kg bw)

Tissues and carcass were analyzed to determine ^{14}C tissue distribution by combustion/LSC. In order to get sufficient sample material for extraction of radioactive residues and metabolic profiling, the total radioactivity values of the individual organs and tissue samples were not determined. Instead, respective sample pools were generated for each test group (i.e. sacrifice time per sex). The following tissues were collected at sacrifice from test animals:

Blood (plasma & RBC)	Liver	Kidney
Leg muscle	GIT and contents	Skin
Perirenal fat	Carcass	

5. Quantitation of Radioactive Residues

Radioactive residues were measured in plasma, urine (including cage rinse i.e. funnel rinse from the Makrolon® metabolism cage), and tissues. The radioactivity of faeces samples was not determined, because they were added at sacrifice to the gastrointestinal tract (GIT). The measurement of the radioactivity in liquid samples was carried out by LSC. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$

was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

6. Quantification and Identification of Metabolites

For metabolic profiling, the urine, plasma, liver, kidney, muscle (leg), and fat (perirenal) samples were pooled across individual animals for a given collection interval for each sex. Liquid chromatography (LC)/mass spectrometry (MS) methods were employed for identification and quantification of the metabolites in each sample. Parent compounds and metabolites were identified by:

- Spectroscopic investigations (LC-MS) of the 0 – 24 h urine from males (test 3) and females (test 6): BYI 02960-difluoroethyl-amino-furanone, isomers 1 and 3 of BYI 02960-OH-gluA, BYI 02960-OH and BYI 02960. Spectroscopic investigation (LC-MS) of the 6 hour plasma from males (test 2) and females (test 5): BYI 02960-DFA, BYI 02960-difluroethyl-amino-furanone, BYI 02960-OH and BYI 02960.
- HPLC comparison of the 0 – 6 h urine sample of males (test 2) and females (test 5) with the radiolabelled reference compounds BYI 02960-difluoroethyl-amino-furanone, and the isomers 1 and 3 of BYI 02960-OH-gluA.
- TLC-co-chromatography of urine and plasma, and extracts from liver, kidney and muscle of males (test 3) and females (test 6) with the radiolabelled reference compound BYI 02960-DFA.
- Comparison of the HPLC-radiochromatograms from all samples of each individual test.
- Comparison of the HPLC-radiochromatograms from the specific samples of male and females: urine, plasma, and extracts from liver, kidney, muscle and fat.

Other peaks or peak groups additionally detected in the HPLC-profiles were designated as "unknown". They were characterised by their behaviour during extraction and clean-up and the retention times in the HPLC-chromatograms.
Parent compound and metabolites were quantified in the original urine and plasma samples and in the conventional acetonitrile/water extracts of organs and tissues by integration of the ^{14}C-signals in the HPLC-chromatograms.

II. RESULTS AND DISCUSSION

A. STORAGE STABILITY

No stability data was reported for the test substance and metabolites in plasma and urine stored at <-18^{o}C, nor in freeze dried samples including faeces/GIT contents, liver, kidney, muscle and fat stored at 4^{o}C.

B. MAIN STUDY

Clinical Observation

Clinical signs were not discussed in the study report.

Recovery
The mean total radiolabel recovery ranged from 95.85% to 98.03% of the administered dose in males (tests 1 to 3), and from 100.3 to 103.7% in females (tests 4 to 6) (Table 3).

Table 3. Tests 1 – 6: Balance of radioactivity in urine and in organs and tissues of male and female rats sacrificed 1 h, 6 h and 24 h after single oral administration.

Percent of total dose administered (mean arith.)						
Test number	**1**	**2**	**3**	**4**	**5**	**6**
Sex	**M**	**M**	**M**	**F**	**F**	**F**
No. of animals	**4**	**4**	**4**	**4**	**4**	**4**
Test period (h)	**1**	**6**	**24**	**1**	**6**	**24**
Excretion						
Urine	6.22	36.53	71.80	8.76	39.70	85.88
Samples						
Plasma	1.09	0.62	0.21	1.27	0.61	0.13
Carcass	38.32	24.47	4.25	51.19	29.57	3.08
Kidneys	1.30	0.73	0.08	1.17	0.63	0.06
Liver	6.80	3.44	0.60	7.30	3.62	0.39
GIT+faeces	27.24	21.18	16.61	13.94	14.26	9.44
Skin	14.64	9.19	2.13	17.54	10.48	1.45
Fat perirenal	0.10	0.04	0.01	0.15	0.09	0.01
Leg muscle	2.31	1.14	0.17	2.34	1.35	0.13
Balance	**98.03**	**97.33**	**95.85**	**103.70**	**100.30**	**100.60**
Body excluding GIT	64.56	39.63	7.45	80.96	46.35	5.26
GIT + faeces	27.24	21.18	16.61	13.94	14.26	9.44
Body	91.80	60.80	24.06	94.91	60.61	14.70

Table excerpted from page 55 of the study report.

Absorption

The study authors did not estimate a final absorbed dose. Excluding the gastro-intestinal tract and its contents, the mean demonstrably absorbed fraction of oral dosing by 24 hours post-dosing was approximately 79% (95.85% minus 16.61%) and 91% (100.6% minus 9.44%) for males and females respectively (Table 3).

Distribution

For both sexes, the highest residue values in organs and tissues were detected at the one hour time-point with approximately 64.6/81.0% (M/F) of the applied dose being in organs and tissues and approximately 27.2/13.9% (M/F) in the GIT and faeces sample. Residue values in organs and tissues declined substantially by 24 hours post dosing to 7.45/5.26% (M/F) of the administered dose (Table 3).

These values indicated a fast distribution of the absorbed radiolabel within the body followed by a quick elimination predominately via urine, with cumulative urinary excretions of 6.2/8.8% (M/F) of the applied dose at one hour and 71.8/85.9% (M/F) by 24 hours.

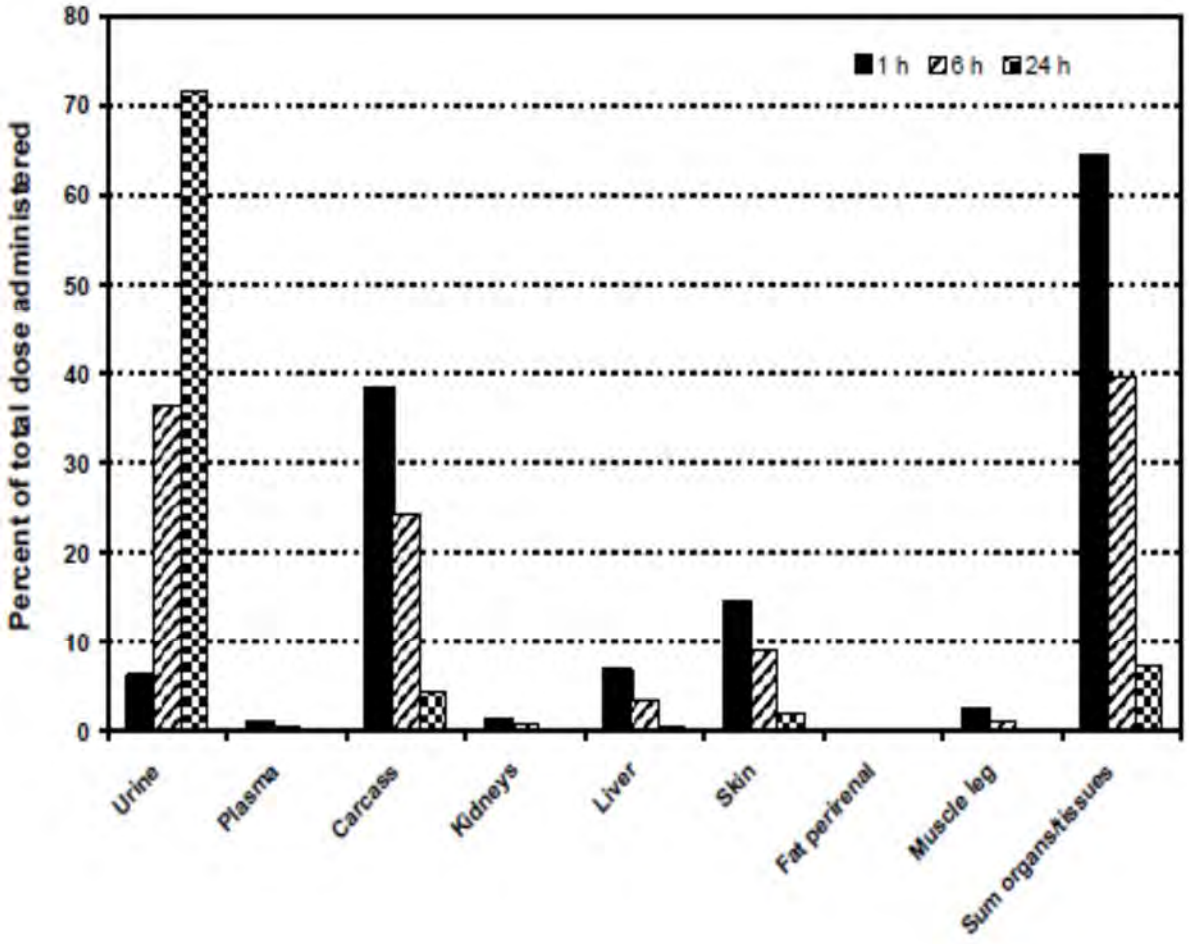


Figure 1: Tests 1 – 3: Total radioactivity in plasma, organs and tissues of male rats sacrificed 1 h, 6 h, and 24 h after single oral dosing.

Figure excerpted from page 108 of the study report.

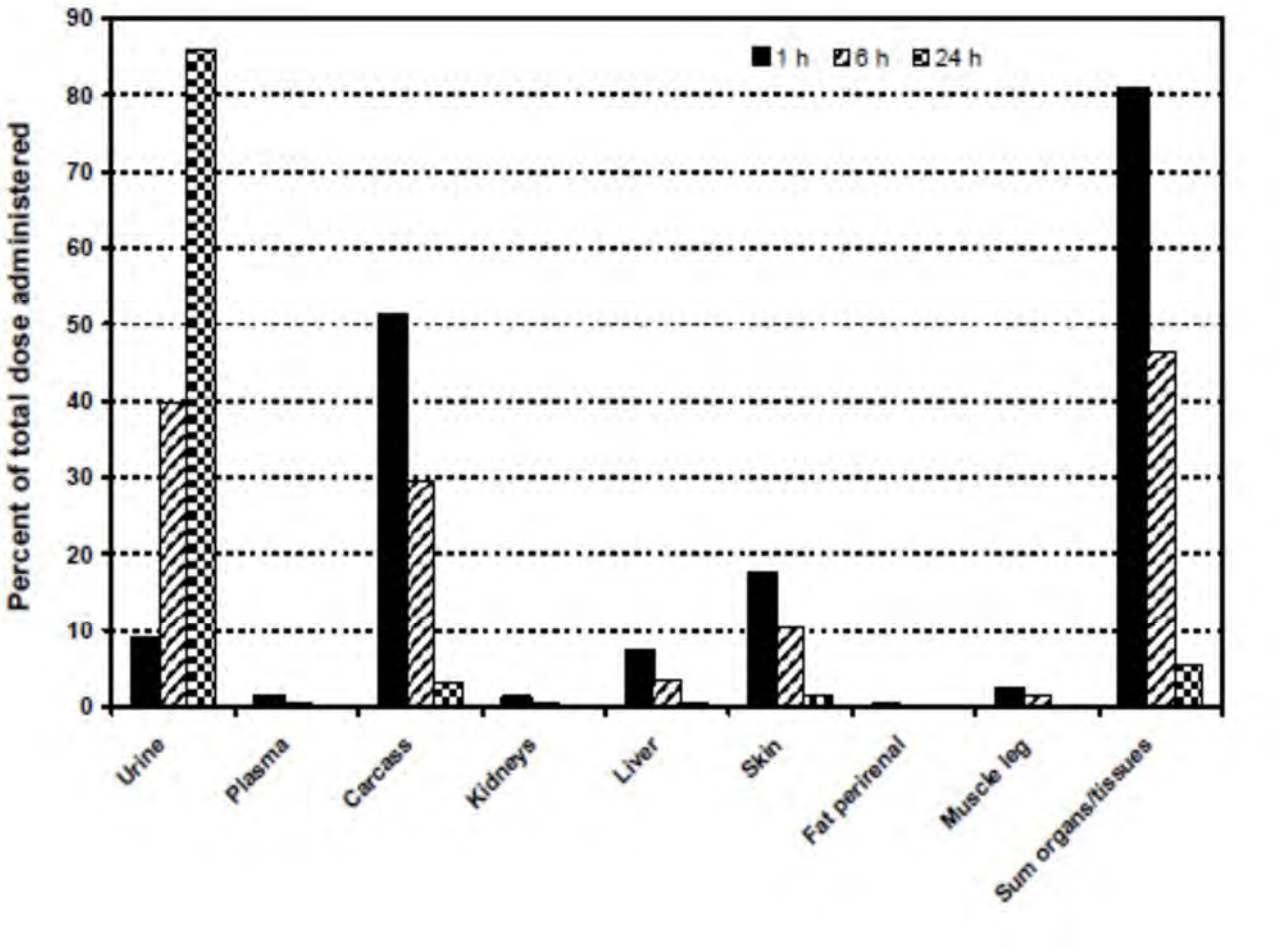


Figure 2. Total radioactivity in plasma, organs and tissues of female rats sacrificed 1 h, 6 h, and 24 h after single oral dosing.

Figure excerpted from page 109 of the study report.

Excretion

Urinary excretion began immediately after dosing and increased until 24 hours to 71.80% of the administered dose in males and 85.88% in females. These results were stated to be in good accordance to the urinary excretion behaviour of all other rat studies (Klempner 2011,

Study No M1824559-4: Koester 2011b, Study No's M1821760-5 and M1821837-0; Weber 2011a/b, Study No's M1824567-3 and M1824560-6).

Metabolism

Urine

In males, the major metabolites of the 0 – 24 hours sample with more than 1% but less than 5% of the dose were identified as BYI 02960-OH-gluA (isomer 1 1.55% and isomer 3 1.72%), BYI 02960-DFA (1.91%) and BYI 02960-difluoroethyl-amino-furanone (3.09%). In females, BYI 02960-DFA was the major metabolite of the 0 – 24 hours sample with more than 1% but less than 5% of the dose were identified as such (1.70%). The prominent metabolite BYI 02960-OH accounted for approx. 12.3% of the dose in males and 6.4% in females. The by far largest component was identified as parent compound (approx. 47.7/76.5% of the dose in M/F.

Identification rates of parent compound and metabolites in the 0 – 24 hours urine sample was 95.1% in males and 99.5% in females of the total radioactive residue (TRR).

Plasma

In plasma from the one hour sample, parent compound comprised of 1.998/2.671 (M/F) mg/kg sample of total plasma radioactivity while DFA (difluoroacetic acid), BYI 02960-difluoroethyl-amino-furanone (males only) and BYI 02960-OH (males only) each accounted for 0.06 mg/kg or less (Table 4). At six hours, parent compound accounted for 1.494/1.661 (M/F) mg/kg of plasma radioactivity (30/40% decreases in M/F) with DFA levels increasing by 6-fold/3-fold (M/F) to 0.348/0.175 mg/kg, respectively. At this timepoint, absolute levels of difluoroethyl-amino-furanone and BYI 02960-OH, both detected only in males, increased by 1.6-fold (0.084 mg/kg) and decreased by 20% (0.045 mg/kg), respectively. An additional two unknown metabolites were detected in only males at 6 hours and were present at 0.024 and 0.018 mg/kg (Table 5). At 24 hours, parent compound levels in plasma had fallen by 80 to 90% of its levels at 1 hour, to reach 0.491/0.343 mg/kg in M/F, respectively. At this timepoint, DFA had increased further to reach 7.7-fold/4.9-fold (M/F) levels at 1 hour, to 0.448/0.28 mg/kg (M/F), respectively, while (in males only) BYI 02960-OH (0.005 mg/kg) and difluoroethyl-amino-furanone (0.007 mg/kg) had fallen to 10% of the levels at 1 hour.

Identification rates of parent compound and metabolites in plasma ranged from 97 to 100% of the TRR in male rats and were 100% for female rats.

Table 4. Radioactive residue of parent compound and metabolites in the plasma of male and female rats

Test No.	**1**	**2**	**3**	**4**	**5**	**6**
Sex	**M**	**M**	**M**	**F**	**F**	**F**
Sacrifice after administration	**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Sampling time	**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
	Equiv. concentration (mg/kg sample)					
Total radioactive residue in plasma pool sample	**2.166**	**1.494**	**0.491**	**2.728**	**1.661**	**0.343**

Compound	*Peak No.*						
Parent	21	1.998	0.976	0.031	2.671	1.486	0.063
DFA	1	0.058	0.348	0.448	0.057	0.175	0.280
unknown	3	---	0.024	---	---	---	---
difluoroethyl-amino-furanone	6	0.052	0.084	0.005	---	---	---
unknown	19	---	0.018	---	---	---	---
BYI02960-OH	20	0.058	0.045	0.007	---	---	---
Total identified		**2.166**	**1.452**	**0.491**	**2.728**	**1.661**	**0.343**
Total characterised*		---	0.042	---	---	---	---
Sum total		**2.166**	**1.494**	**0.491**	**2.728**	**1.661**	**0.343**

*Peaks were characterised based on their retention time in HPLC-analysis: none of them accounted for >0.024 mg/kg.
'DFA' denotes difluoroacetic acid.

Data excerpted from page 59 of the study report.

Liver

In liver at one hour, parent compound was present at 3.90/5.519 (M/F) mg/kg sample, while DFA, BYI 02960-difluoroethyl-aminofuranone and BYI 02960-OH were all present at 0.187 mg/kg or less in both sexes. An additional unknown metabolite (peak No. 19) was seen in both sexes (0.042/0.021 mg/kg, M/F) at this timepoint, with a further 3 unknown metabolites (peak No. 2, 11 and 18) being present in females only and all at 0.019 mg/kg or less (Table 5). At six hours, levels of parent compound fell in both sexes by 50% to 1.903/2.977 mg/kg for M/F. Increases were in both sexes at this timepoint for DFA levels (3.7-fold/3.5-fold for M/F), while BYI 02960-difluoroethyl-amino-furanone levels remained relatively stable (20% increase in males, 20% decrease in females) and BYI 02960-OH decreased by 20/40% (M/F). BYI 02960-OH-gluA (isomer 1) was detected in males at 6 hours only, at 0.021 mg/kg. Additionally at six hours, unknown metabolites with peak No's of 2 (males only), 3 (males only), 10 (males only), 11, and 19 were observed at levels of 0.012 to 0.021 mg/kg and were absent at 24 hours. At 24 hours, the parent compound level decreased substantially to less than 2% of levels at 1 hour (0.084 mg/kg for both sexes), while DFA levels increased to 4.4-fold/4.1-fold the levels at 1 hour (0.218/0.136 mg/kg for M/F), and levels of BYI 02960-OH fell to less than 8% of 1 hour levels (0.014/0.005 mg/kg for M/F). Unknown metabolites detected at 24 hours in liver samples were confined to females, with previously undetected compounds of peak No.'s 15 and 16 being present at 0.002 mg/kg each (Table 5).

Identification rates of parent compound and metabolites in liver ranged from 86 to 98% of the TRR for males and 92.6 to 98.9% for females.

Table 5. Radioactive residue of parent compound and metabolites in the liver of male and female rats

Test No.	**1**	**2**	**3**	**4**	**5**	**6**
Sex	**M**	**M**	**M**	**F**	**F**	**F**
Sacrifice after administration	**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Sampling time	**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
	Equiv. concentration (mg/kg sample)					

Total radioactive residue in liver pool sample		**4.281**	**2.507**	**0.367**	**5.770**	**3.225**	**0.243**
Parent	*Peak No.*						
	21	3.900	1.903	0.084	5.519	2.977	0.084
DFA	1	0.049	0.183	0.218	0.033	0.116	0.136
unknown	2	---	0.012	---	0.019	---	---
unknown	3	---	0.012	---	---	---	---
difluoroethyl-amino-furanone	6	0.070	0.086	---	0.022	0.018	---
OH-gluA(isomer1)	9	---	0.021	---	---	---	---
unknown	10	---	0.013	---	---	---	---
unknown	11	---	0.013	---	0.017	0.	---
unknown	15	---	---	---	---	---	0.002
unknown	16	---	---	---	---	---	0.002
unknown	18	---	---	---	0.014	---	---
unknown	19	0.042	0.031	---	0.021	0.010	---
BYI02960-OH	20	0.187	0.150	0.014	0.106	0.063	0.005
Total identified		**4.206**	**2.343**	**0.316**	**5.969**	**3.190**	**0.225**
Total characterised*		0.042	0.081	---	0.055	0.010	0.004
Total identified or characterised		4.248	2.434	0.316	5.751	3.200	0.229
Solids (unextractable)		0.023	0.065	0.044	0.012	0.015	0.011
Not analysed samples		0.010	0.018	0.008	0.007	0.010	0.002
Sum total**		**4.281**	**2.507**	**0.367**	**5.770**	**3.225**	**0.243**

*Peaks were characterised based on their retention time in HPLC-analysis: none of them accounted for >0.042 mg/kg
** Sum total = Total identified or characterised + solids (unextractable) + not analysed samples
'DFA' denotes difluoroacetic acid.

Data excerpted from page 62 of the study report.

Kidney

In the kidney at one hour, parent compound comprised 4.268/4.695 mg/kg (M/F) sample, while other known metabolites consisted of DFA, BYI 02960-difluoroethyl-amino-furanone (males only) and BYI 02960-OH at 0.341 mg/kg or less in both sexes (Table 6). At six hours, levels of parent compound decreased by approx. 50% in both sexes to 2.108/2.530 mg/kg (M/F), while levels of DFA by 4.2-fold/3.5-fold in M/F and levels of BYI 02960-OH decreased by 2% (M) to 25% (F). In males, difluoroethyl-amino-furanone increased by 1.8-fold at this timepoint to reach 0.153 mg/kg, and unknown metabolites with peak No's of 4, 5 and 19 were present at 0.036 mg/kg or less and were not seen at the other two timepoints (Table 7). At 24 hours, levels of parent compound decreased substantially to 2% of levels at 1 hour, falling to 0.082/0.102 mg/kg (M/F), while DFA levels increased further to reach 4.8-fold/3.8-fold levels at 1 hour (0.200/0.144 mg/kg for M/F) (Table 6).

Identification rates of parent compound and metabolites in kidney ranged from 95 to 100% of the TRR for males and 96 to 100% for females.

Table 6. Radioactive residue of parent compound and metabolites in the kidney of male and female rats

Test No.	**1**	**2**	**3**	**4**	**5**	**6**

Sex		**M**	**M**	**M**	**F**	**F**	**F**
Sacrifice after administration		**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Sampling time		**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Total radioactive residue in kidney pool sample		**4.747**	**2.896**	**0.317**	**4.901**	**2.795**	**0.267**
Parent	*Peak No.*	**Equiv. concentration (mg/kg sample)**					
	21	4.268	2.108	0.082	4.695	2.530	0.102
DFA	1	0.042	0.176	0.200	0.038	0.133	0.144
unknown	4	---	0.036	---	---	---	---
unknown	5	---	0.019	---	---	---	---
difluoroethyl-amino-furanone	6	0.084	0.153	---	---	---	---
unknown	19	---	0.034	---	---	---	---
BYI02960-OH	20	0.341	0.334	0.020	0.155	0.115	0.011
Total identified		**4.735**	**2.771**	**0.303**	**4.888**	**2.778**	**0.257**
Total characterised*		---	0.088	---	---	---	---
Total identified or characterised		4.735	2.860	0.303	4.888	2.778	0.257
Solids (unextractable)		0.007	0.022	0.014	0.005	0.008	0.010
Not analysed samples		0.005	0.014	n.q	0.007	0.009	n.q
Sum total**		**4.747**	**2.896**	**0.317**	**4.901**	**2.795**	**0.267**

*Peaks were characterised based on their retention time in HPLC-analysis: none of them accounted for >0.036 mg/kg.
** Sum total = Total identified or characterised + solids (unextractable) + not analysed samples
'DFA' denotes difluoroacetic acid.

Data excerpted from page 66 of the study report.

Muscle (leg)

In muscle at one hour, parent compound comprised 2.201/2.827 mg/kg sample (M/F), while DFA, BYI 02960-difluoroethyl-aminofuranone and BYI 02960-OH each were present at 0.061 mg/kg or less in both sexes (Table 7). At 6 hours, levels of parent compound decreased by approx. 50% in both sexes (1.097/1.568 mg/kg for M/F), while levels of DFA increased by 6-fold/3-fold in M/F to 0.129/0.07 mg/kg (M/F), difluoroethyl-amino-furanone increased by 1.4/1.2-fold (M/F) (0.070/0.019 mg/kg for M/F), and BYI 02960-OH fell by 20 to 40% (0.049/0.021 mg/kg for M/F). At 24 hours, levels of parent compound for both sexes fell to approx. 2 to 3% of levels at 1 hour (0.075/0.055 mg/kg for M/F), while DFA levels increased by 5.9-fold/4.7-fold (M/F) of 1 hour levels to reach 0.124/0.108 mg/kg (M/F). The only other metabolite detected in muscle at this timepoint was BYI02960-OH, at 0.003 mg/kg in females.

Identification rates of parent compound and metabolites in muscle ranged from 95 to 100% of the TRR for males and 99 to 100% for females.

Table 7. Radioactive residue of parent compound and metabolites in muscle of male and female rats

Test No.	**1**	**2**	**3**	**4**	**5**	**6**
Sex	**M**	**M**	**M**	**F**	**F**	**F**

Sacrifice after administration		**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Sampling time		**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Total radioactive residue in muscle pool sample		**2.336**	**1.352**	**0.208**	**2.902**	**1.682**	**0.167**
Parent	*Peak No*	**Equiv. concentration (mg/kg sample)**					
	21	2.201	1.097	0.075	2.827	1.568	0.055
DFA	1	0.021	0.129	0.124	0.023	0.070	0.108
difluoroethyl-amino-furanone	6	0.050	0.070	---	0.016	0.019	---
BYI02960-OH	20	0.061	0.049	---	0.035	0.021	0.003
Total identified		**2.332**	**1.346**	**0.198**	**2.901**	**1.678**	**0.166**
Total characterised*		---	---	---	---	---	---
Total identified or characterised		2.332	1.346	0.198	2.901	1.678	0.166
Solids (unextractable)		0.001	0.002	0.002	0.001	0.001	0.001
Not analysed samples		0.003	0.004	0.007	n.q.	0.003	n.q.
Sum total**		**2.336**	**1.352**	**0.208**	**2.902**	**1.682**	**0.167**

*Peaks were characterised based on their retention time in HPLC-analysis: none
**Sum total = Total identified or characterised + solids (unextractable) + not analysed samples
'DFA' denotes difluoroacetic acid.

Data excerpted from page 70 of the study report.

Perirenal fat

In perirenal fat at one hour, parent compound was present at 0.911/1.079 mg/kg sample (M/F) and was the sole radioactive constituent at this timepoint (Table 8). At six hours, levels of parent compound fell by approx. 60/40% (M/F) of one hour levels, while DFA was present at 0.051/0.031 mg/kg (M/F) and (males only) unknown Peak No's 19 (0.035 mg/kg) and 20 (0.018 mg/kg) were also detected at this time point (Table 9). At 24 hours, parent compound fell further to 4% or less of 1 hour levels in both sexes (0.03/0.013 mg/kg for M/F), while DFA levels increased in males by 1.45-fold and remained stable in females (0.074/0.032 mg/kg for M/F). No other metabolites were detected in perirenal fat (Table 9).

Identification rates of parent compound and metabolites in muscle ranged from 93 to 100% of the TRR for males and 96 to 100% for females.

Table 8. Radioactive residue of parent compound and metabolites in fat extracts of male and female rats

Test No.		**1**	**2**	**3**	**4**	**5**	**6**
Sex		**M**	**M**	**M**	**F**	**F**	**F**
Sacrifice after administration		**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Sampling time		**1 h**	**6 h**	**24 h**	**1 h**	**6 h**	**24 h**
Total radioactive residue in fat pool sample		**0.911**	**0.493**	**0.108**	**1.079**	**0.655**	**0.047**
Parent	*Peak No*	**Equiv. concentration (mg/kg sample)**					
	21	0.911	0.389	0.030	1.079	0.624	0.013
DFA	1	---	0.051	0.074	---	0.031	0.032
unknown	19	---	0.035	---	---	---	---
unknown	20	---	0.018	---	---	---	---
Total identified		**0.911**	**0.458**	**0.104**	**1.079**	**0.654**	**0.045**

Total characterised*	---	0.035	---	---	---	---
Total identified or characterised	0.911	0.492	0.104	1.079	0.654	0.045
Solids (unextractable)	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
Not analysed samples	n.q	n.q	0.003	n.q	n.q	0.002
Sum total**	**0.911**	**0.493**	**0.108**	**1.079**	**0.655**	**0.047**

*Peaks were characterised based on their retention time in HPLC-analysis: none accounted for >0.035 mg/kg
** Sum total = Total identified or characterised + solids (unextractable) + not analysed samples
'DFA' denotes difluoroacetic acid.

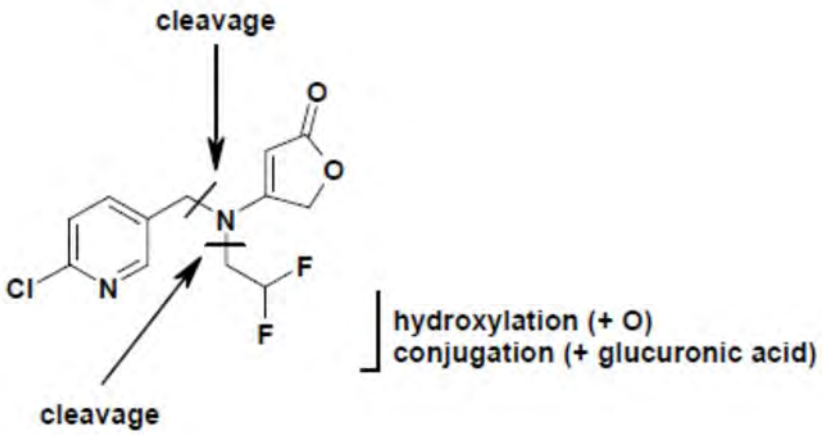
Data excerpted from page 74 of the study report.

Overall, BYI02960 was metabolised extensively. Metabolic reactions took place at least at three different structural positions of the test compound. The majority of components were identified (approximately 97 – 100% of TRR in plasma, and in extracts of organs and tissues as well as ≥ 95% of radioactivity in urine samples). In the 24 hours samples of plasma, and organs and tissues DFA was by far the largest metabolite accounting for more than 50% of the TRR. For all other identified metabolites, the values were less than 10% of the TRR. The percentage-values of the parent compound in these samples ranged from 6 to 38% of the TRR. In the respective urine sample pools, the parent compound was the largest radioactive component (approximately 48% of the dose in males and 77% in females). The metabolism was qualitatively similar in male and females. The differences occurred however quantitatively. The degradation of the parent compound to the different metabolites was significantly higher in males compared to females.

The principal metabolic reactions of [ethyl-4-^{14}C]BYI02960 in the rat were:

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

The following figure shows schematically the positions in the molecule, which are involved in the metabolic reactions:



The proposed biotransformation pathway of [ethyl-4-^{14}C]BYI02960 is presented in Figure 1 below.

The metabolism results with [ethyl-4-^{14}C]BYI02960 are in good accordance with those obtained from the ADME rat study by Weber (2011b) and organ metabolism study by Koester (2012b; Study No. M1824574-1) conducted with [furanone-4-^{14}C]BYI02960.

Figure 1. Proposed metabolic pathway of [ethyl-1-^{14}C]BYI 02960

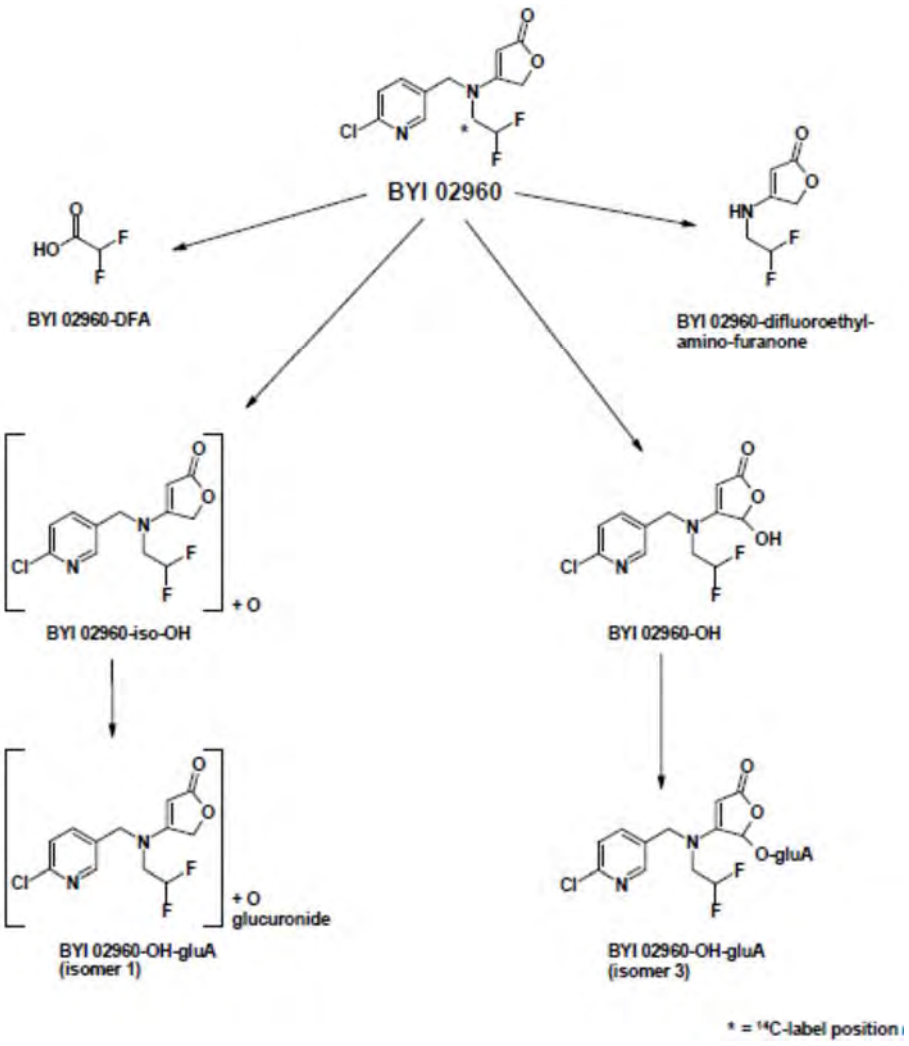


Figure excerpted from page 15 of the study report.

III. CONCLUSION

The kinetic and metabolic behaviour of [ethyl-1-^{14}C]BYI02960 in Wistar rats has the following characteristics:

- The distribution of the radioactivity within the central compartments (i.e. blood, liver, kidney, muscle and fat) of the body was fast and showed a distinctive preference towards the liver and kidney as the main organs responsible for metabolism and excretion.
- The highest TRR-values were measured in plasma, and in organs and tissues at one hour after dosing, and decreased significantly over 24 hours. There was no indication of persistence or accumulation of the test substance or its metabolites.
- Parent compound, major and several minor metabolites were identified in all samples. Identification rates were high with ≥ 95% of radioactivity in urine and approximately 93 – 100% of TRR in plasma, and organs and tissues.
- The metabolic transformation of BYI02960 was principally oxidative and took place at least at three different structural positions.
- DFA was the by far largest metabolite in plasma, and in organs and tissues. The parent compound was on the other hand the largest radioactive component in the urine samples. The metabolic pattern was in good accordance with that obtained from the corresponding ADME rat study by Weber (2011a; Study No M1824567-3).
- A clear sex difference was observed for the extent of metabolism, being higher in male than in females. The unchanged parent compound was more pronounced in females than in males.

Many examples of sex differences of metabolism in rats have been reported. A very important and general observation is the approximately threefold difference in the activity of hepatic microsomal monooxygenase (cytochrome P-450) in male rats compared to females.

Overall, based on the results obtained the depletion of radioactive residues from the organs and tissues of male and females and metabolism of [ethyl-1-^{14}C]BYI02960 are assumed as adequately understood.

Primary Reviewer: Australia
Secondary Reviewer: US EPA

Report: Repetto M. (2011). BYI 02960 28-day Immunotoxicity Study in the Female Wistar Rat by Dietary Administration. Bayer S.A.S., Bayer CropScience [355, rue Dostoïevski, BP 153, 06903 Sophia Antipolis Cedex. France]. Study ID: SA 10353. Bayer CropScience Report No. M-414754-01-2. 22 September 2011. MRID 48844148. PMRA 2239155. Unpublished.

Sponsor: Bayer CropScience AG, Alfred Nobel Str. 50, 40789 Monheim, Germany

Guidelines: OPPTS 870.7800 (1998)

Deviations: None

GLP: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were presented in the report.

Executive summary:
In an immunotoxicity study (MRID 48844148), BYI 02960 (96.2% a.i. batch# 2009-000239) was administered to seven week old female Wistar, Rj:WI (IOPS HAN) rats in groups of 10/dose at concentrations of 0, 125, 600, or 3000 ppm (0, 10, 50, or 230 mg/kg bw/d) for 30 days. A positive control group consisting of 10 female Wistar, Rj:WI (IOPS HAN) rats received oral gavage doses of 3.5 mg/kg bw/d cyclophosphamide at 5 mL/kg bw dosing volumes for 28 days.

On Day 26, the test animals were immunized with an injection of sheep red blood cells (SRBC). On study Day 30, blood samples were collected from the test animals and serum samples were assayed for their concentration of SRBC-specific IgM antibodies to provide a quantitative assessment of humoral immune response by ELISA. Additional parameters that were evaluated include mortality, clinical signs of toxicity, bodyweight, bodyweight gain, food consumption, selected organ weights (*i.e*. spleen, and thymus), and macroscopic examination of all major organs, tissues and body cavities.

There were no observable test substance related clinical signs, and premature mortality was confined to euthanasia of one animal at 600 ppm due to forelimb automutilation on study day 8. As this was unaccompanied by other necropsy findings, it was considered unrelated to the test substance.

Overall group mean bodyweight gains at 3000 ppm over study days 1 to 29 were 23% lower than that in controls ($p<0.05$), resulting in 6% lower mean terminal bodyweight than controls without statistical significance ($p > 0.05$). Decreases in food consumption of ≤ 34% (week 1) relative to controls were seen at 3000 ppm throughout the study period and were likely linked to changes observed in bodyweight gain.

Necropsy findings in negative control and test substance groups were unremarkable, whereas atrophied and/or small (5 or 6/10 animals) spleens were observed in cyclophosphamide-dosed positive control animals.

While there was a dose-related decreasing trend in absolute spleen weights relative to controls in treated animals (2%, 4% and 6% decreases at 125, 600 and 3000 ppm respectively), relative (to bodyweight) spleen weights were essentially unchanged at 3000 ppm (+1% increase). Similarly, absolute thymus weights at 3000 ppm were decreased by 4% relative to controls, but relative (to

bodyweight) thyroid weights were unaffected. Positive control animals had 32 to 34% ($p \leq 0.01$) decreases in mean absolute and relative spleen weights, and 27 to 28% ($p \leq 0.01$) decreases in absolute and relative thymus weights, respectively.

The SRBC-specific IgM ELISA data showed a 55% increase from controls at 125 ppm, 15% decrease at 600 ppm, and an 18% decrease at 3000 ppm, with no findings being statistically significant ($p > 0.05$). Based on the lack of dose dependency and absence of statistical significance, overall there were no indications of test substance immunosuppressive potential up to and including the highest dose tested of 3000 ppm (~230 mg/kg bw/d). The positive control performed as expected, with a 93% decrease ($p \leq 0.01$) in anti-SRBC IgM antibody concentrations.

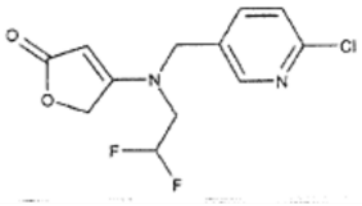
The immunotoxicity NOAEL is the highest dose tested of 3000 ppm (230 mg/kg bw/d), based on an absence of test substance related immunotoxicity findings at this dose.

This study is fully reliable (acceptable/guideline) and satisfies the guideline requirements (OPPTS 870.7800) for an immunotoxicity study in rats.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   Description: Beige powder
   Lot/Batch #: 2009-000239
   Purity: 96.2% (various methods; date of analysis 14 January 2011)
   Compound Stability: Found to be stable in prepared diet over duration of study. Expiry if stored at 20±10ºC stated to be two years from date of analysis.
   CAS#: Not reported
   Structure:



2. Vehicle and Positive Control
   Vehicle: Basal diet with acetone solvent

   Positive control: Oral gavage dosing with cyclophosphamide in water vehicle (Source: Sigma-Aldrich, USA. Batch No's. B 068K1131; 98% purity by HPLC, or 120M1253; 100.6% purity by HPLC, stored in light resistant containers at 5±3ºC when not in use).

3. Test Animals:

Species:	Rat (female only)	
Strain:	Wistar, Rj:WI (IOPS HAN)	
Acclimation period	12 days	
Bodyweight at dosing:	165 to 206 g	
Age at dosing:	7 weeks	
Source:	R. Janvier, Le Genest St Isle, France	
Housing:	Individually in suspended, stainless steel, wire-mesh cages	
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 (Scientific Animal Food and Engineering, Augy, France), *ad libitum*	
Water:	Filtered and softened tap water, *ad libitum*	
Environmental Conditions:	Temperature:	20 to 24ºC
	Humidity:	40 to 70%
	Air Changes:	10 to 15/hour
	Photoperiod:	12 hrs dark / 12 hrs light

B. STUDY DESIGN:

1. Experimental
   23 March 2011 – 23 May 2011

2. Animal Assignment and treatment

Four groups of 10 female Wistar, Rj:WI (IOPS HAN) rats/dose were administered BYI 02960 in feed daily at 0, 125, 600, or 3000 ppm in feed daily for 30 days, with mean doses determined to be approximately 10, 50, and 230 mg/kg bw/day. Additionally, a positive control group of 10 female Wistar, Rj:WI (IOPS HAN) rats were administered the positive control cyclophosphamide at 3.5 mg/kg bw/day at a 5 mL/kg bw dose volume daily for 30 days (Table KIIA 5.10.2/01).

The study author stated that Wistar rats were chosen for this study as the Wistar rat has been used extensively in toxicity evaluation studies and is a rodent model for immunotoxicity studies. Furthermore, the rat demonstrated greater sensitivity to general systemic toxicities compared to the mouse in previous toxicity studies conducted with BYI 02960. Additionally, the study author stated that females were used since changes in thymus and spleen weights were observed in reproduction studies using BYI 02960 (Milius, 2011; MRID 48844119).

Animals were assigned to dose groups by computerised, stratified randomisation so that body weights were within 20% of the mean body weight on the day of randomisation. Animal housing and husbandry were in accordance with the provisions of the *Guide to the Care and Use of Laboratory Animals* (USPHS-NIH Publication No. 86-23) and "Le Guide du Journal Officiel des Communautes Europeennes L358, 18 Decembre 1986, n° 86/609/CEE du 24 Novembre 1986".

Table KIIA 5.10.2/01. Study Design

Group	Dosing method	No./females/dose	Nominal dietary dose (ppm)	Achieved mean dose level (mg/kg bw/d)
BYI 02960				
1	Dietary	10	0	0
2	Dietary	10	125	10
3		10	600	50
4	Dietary	10	3000	230
Cyclophosphamide (positive control)				
5	Oral (gavage)	10	-	3.5

Data excerpted from pages 18 and 27 of the study report.

3. Diet Preparation and Analysis (including positive control)

BYI 02960

The required amount of the test material ground to a fine powder and solvated in acetone was incorporated into the diet by dry mixing to achieve the nominal dietary concentrations at each dosing level. For the negative control group, a control formulation was prepared by adding an equivalent volume of acetone into the diet. There was one preparation for each concentration which was prepared once at the beginning of the study. When not in use, prepared diet formulations were stored at room temperature.

The stability of BYI 02960 in the diet was evaluated at 3000 ppm in prepared diet over 34 days storage at room temperature. The stability of BYI 02960 in the diet was shown to be 99% of day 1 measured concentrations (2835.5 ppm or 95% of nominal). Additionally, the stability of BYI 02960 in the diet was reported from the evaluation undertaken in the chronic toxicity and carcinogenicity study in Wistar rats (Garcin, 2012; MRID 48844123). In this study, the stability

of BYI 02960 in the diet was evaluated at 70 and 2500 ppm after storage at room temperature for 110 days, and was shown to be 89 – 94% of the nominal concentration.

Achieved concentrations and homogeneity analysis was undertaken on 4 samples/concentration at 125 and 3000 ppm and found to be between 90% and 96% of nominal concentrations respectively. An additional analysis of the 600 ppm nominal feed prepared found an achieved concentration of 561 ppm (94%; further details not provided).

Cyclophosphamide

There were two preparations of cyclophosphamide in sterilized water used during the study, and due to an insufficient volume of the second formulation, it was stated by the study author that 23 ml were taken from the second formulation (F2) of the study SA 10354 (not the same batch but the same concentration). When not in use, the preparations were stored in air-tight light resistant containers at approximately +5°C (±3°C) when not in use.

The stability of cyclophosphamide in vehicle was determined in this study. The study author reported the stability had been demonstrated in a previous immunotoxicity study (Odin-Feurtet, 2009; Study No. SA09023) at concentrations of 1 and 3 g/L for a time period which covers the period of storage and usage for the current study, and also in a further immunotoxicity study (Odin-Feurtet, 2009; Study No. SA10354) at concentrations of 0.1 and 1 g/L for a time period which covers the period of storage and usage for the current study (neither of these studies were submitted for evaluation).

The homogeneity of cyclophosphamide in vehicle was verified on the first formulation in the current study.

It was reported that achieved concentrations and homogeneity ranged from 96 and 99% of the nominal concentration

4. Statistics

Bodyweights & bodyweight gain, food consumption, organ weights and immunological parameters were subjected to statistical analyses. Mean and standard deviation were calculated for each group. The analyses were undertaken on group means using Path/Tox System V4.2.2 software (Module Enhanced Statistics), and statistical significance was defined at $p \leq 0.05$ or $p \leq 0.01$ levels.

Terminal bodyweights and organ weights

For terminal bodyweight and organ weight parameters of test substance dosed animals, a Bartlett Test was conducted and if not significantly ($p \geq 0.05$) different from the controls the data were subjected to an ANOVA test. If the ANOVA was not significant ($p > 0.05$), no further analyses was undertaken, otherwise a 2-sided Dunnett Test was conducted.

If the initial Bartlett Test was significantly (*i.e.*, p was ≤ 0.05) different from the controls, a Kruskal-Wallis test was conducted, and if it was not significant ($p > 0.05$), no further analyses was undertaken, otherwise a 2-sided Dunn Test was conducted.

For bodyweight parameters of cyclosphosphamide dosed animals, an F-test was conducted and if significantly (≤ 0.05) different to controls the data were subjected to a Modified 2-sided T-test, otherwise they were subjected to a standard 2-sided T-test.

Bodyweights and food consumption/day
For bodyweight and food consumption per day values of test substance dosed animals collected during the treatment period, an initial Bartlett test was undertaken and if not significantly ($p>0.05$) different from controls, was subjected to an ANOVA test. If the ANOVA test result was not significant ($p>0.05$), no further analysis was undertaken, otherwise the data were subjected to a 2-sided Dunnett Test.

If the initial Bartlett test was significantly ($p\leq0.05$) different from controls, the data was log transformed and subjected to a follow-up Bartlett Test. If the follow-up Bartlett Test was significantly (*i.e.*, $p\leq0.05$) different to controls, a Kruskal-Wallis test was conducted, and if it was not significant ($p>0.05$) no further analyses was undertaken, otherwise a 2-sided Dunn Test was conducted. If the follow-up Bartlett Test on log transformed data was not significantly (*i.e.*, $p>0.05$) different to controls, an ANOVA test on log transformed data was conducted and if the result was not significant ($p>0.05$), no further analysis was undertaken, otherwise the log transformed data were subjected to a 2-sided Dunnett Test.

For bodyweight and food consumption per day values of cyclophosphamide dosed animals collected during the treatment period, an initial F-test was conducted and if not significant (i.e., $p>0.05$) the data was subjected to a standard 2-sided T-test. If the initial F-test was significant ($p\leq0.05$) the data was log transformed and subjected to a follow-up F-test. If the follow up test was significant ($p\leq0.05$), the data was subjected to a Modified 2-sided T-test, otherwise the log-transformed data was subjected to a standard 2-sided T-test.

Immunological parameters
For immunological parameters of the test substance group, a Kruskal-Wallis Test was conducted against controls and if significant ($p\leq0.05$) the data were subjected to 2-sided Dunn Test, otherwise no further analysis was undertaken. For immunological parameters comparison between negative controls and cyclophosphamide positive control, a two-sided Mann-Whitney test was conducted.

C. METHODS

1. 1. Observations
Animals were observed at least twice daily for mortality and morbidity on weekdays and once daily on weekends. Clinical observations were undertaken at least once daily, and the nature, onset, severity, reversibility, and duration of clinical signs were recorded. A detailed physical examination was performed prior to study commencement and at least weekly during the study.

Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose faeces.

2. Bodyweight
All animals were weighed at study initiation and at necropsy, and on a weekly basis over the study period. Body weight change was calculated for each week and for the total study duration.

3. Food consumption, Food efficiency, and Daily intake
Food consumption was recorded on a weekly basis for all animals. Daily intake was calculated from food consumption and body weight data. Food use efficiency estimates were not provided.

4. Humoral immune function, gross pathology and organ weights
On study day 26 animals were immunized via intravenous injection into the lateral tail vein with 0.5 mL of 5×10^8 sheep red blood cells (SRBC)/mL (BioMérieux, address not reported) under isoflurane anaesthesia.

Four days after SRBC injection (study day 30), the animals were sacrificed by exsanguination under deep anesthesia (inhalation of isoflurane). Blood (0.5 mL) was collected from each animal at the sacrifice from the retro-orbital venous plexus, while the animal was under anesthesia. The blood from each rat was processed to serum and frozen at approximately -74°C until analyzed. Spleen and thymus from treated groups were weighed and discarded without further evaluation. Humoral immune function was evaluated by examining serum from individual test substance treated animals for SRBC-specific IgM levels with an enzyme-linked immunosorbent assay (ELISA).

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical Signs of Toxicity

No treatment related clinical signs of systemic toxicity were observed

2. Mortality

Premature mortality was confined to one animal at 600 ppm, which was found to have automutilation of both forelimbs on study Day 8 and was therefore sacrificed for animal welfare reasons. Necropsy findings in this animal were unremarkable. This mortality was not considered to be treatment related.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 3000 ppm, mean body weight from study day 8 to 29 was decreased by 6 to 11% when compared to controls (data not shown), with the finding being statistically significant at the $p \leq 0.01$ level on days 1 to 8 and at the $p \leq 0.05$ level on days 8 to 15. At termination, mean body weight was decreased by 6% (not statistically significant) compared to controls. Additionally at 3000 ppm, there was no mean body weight gain on study days 1 to 8 (versus gains of 21 g, approximately 11% of initial body weight in controls), which was associated with decreased food consumption (see below). Body weight gain was the similar to control thereafter. However, overall mean body weight gains at 3000 ppm over study days 1 to 29 were 23% lower (statistically significant) compared to controls (Table KIIA 5.10.2/02).

No treatment related effect was seen on body weight at 600 and 125 ppm. Compared to controls, decreases in mean body weight gain (not statistically significant) were seen intermittently over the study period, with overall decreases of 5% and 4% observed at 600 and 124 ppm respectively. These body weight gain findings at 600 and 125 ppm were not considered to be toxicologically significant (Table 2).

No treatment related effect was seen on mean body weight with the positive control cyclophosphamide. Variable decreases were seen in mean body weight gain over the dosing period, with an overall decreased body weight gain of 12%. None of these decreases were statistically significant.

Table KIIA 5.10.2/02. Mean body weight gains (g/day) and selected mean body weights (g)

Body weight gains (g/day)	Dose (ppm)				Positive
	0	125	600	3000	control
Days 1 to 8	3±1	2.7±0.8 (-10%)	1.9±3 (-37%)	**0.0±0.6** (-100%)**	2.6±0.8 (-13%)
Days 9 to 15	1.9±0.6	2±0.7 (5%)	2±1 (5%)	2.5±0.5 (32%)	1.7±1.2 (-11%)
Days 16 to 22	2±0.9	2.2±0.7 (10%)	2.3±0.5 (15%)	2.3±0.5 (15%)	1.9±0.7 (-5%)
Days 23 to 29	1.4±0.9	1.1±0.8 (-21%)	0.7±0.6 (-50%)	1.5±0.5 (7%)	0.9±0.6 (-36%)
Total gains Days 1 to 29 (g)	57±10	55±5 (-4%)	54±16 (-5%)	**44±9* (-23%)**	50±7 (-12%)
Day 1 bodyweight (g)	*182±10*	*182±8* (0%)	*182±11* (0%)	*181±8* (-1%)	*182±13* (0%)
Day 29 bodyweight (g)	*240+17*	*237+12 (-1%)*	*237+23 (-1%)*	*225+15 (-6%)*	*232+16 (-3%)*

* denotes p<0.01 or ** p≤0.01. Data excerpted from pages 48 to 60 of the study report.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

At 3000 ppm, compared to controls a statistically significant decrease in mean daily food consumption of 34% was seen in study week 1. For the remaining weeks, decreases of 9 to 13% were observed (not statistically significant) for mean daily food consumption, with an overall mean decrease of 16% (not statistically significant).

No treatment related effect was seen on food consumption at 600 and 125 ppm. Compared to controls, decreases in mean food consumption (not statistically significant) were seen intermittently over the study period, with overall decreases of just 3% observed at both dose levels (Table KIIA 5.10.2/03).

No treatment related effect was seen on food consumption with the positive control cyclophosphamide.

Food use efficiency was not calculated.

Table KIIA 5.10.2/03. Food consumption (g/day)

Food consumption (g/day)	Dose (ppm)				Positive
	0	125	600	3000	control
Days 1 to 8	18.4±1.3	17.7±2.6 (-4%)	17±5.1 (-8%)	**12.2±1.4** (-34%)**	17.7±1.7 (-4%)
Days 9 to 15	18.7±1.1	17.8±1.6 (-5%)	18.8±2.1 (1%)	17±1.7 (-9%)	17.9±1.6 (-4%)
Days 16 to 22	19.5±2.8	20.2±5.8 (4%)	18.9±2.3 (-3%)	16.9±1.3 (-13%)	17.7±1.2 (-9%)
Days 23 to 29	18.3±1.6	17.3±1.4 (-5%)	17.9±2.3 (-2%)	16.6±1.5 (-9%)	17.5±1.1 (-4%)

** denotes a value that is statistically significant at the p<0.01 level. Data excerpted from page 63 and 65 of the study report.

D. HUMORAL IMMUNE FUNCTION, GROSS PATHOLOGY, AND ORGAN WEIGHTS

1. HUMORAL IMMUE FUNCTION

The high mean anti-SRBC IgM concentration observed in the control group confirmed the sensitization of the animals. Compared to controls, a 55% increase and a 15% and 18% decrease were seen in mean anti-SRBC IgM concentration at 125, 600 and 300 ppm respectively, with none of the findings being statistically significant (Table 4).

Examination of the lowest value within each group showed a dose-related decreasing trend of 17708, 9092, and 3672 U/mL for anti-SRBC IgM concentration at 125, 600, and 3000 ppm respectively, with corresponding maximum values of 146500, 105300, and 90028 U/mL also suggesting a dose-related decreasing trend. However, noting the high inter-individual variability within dose groups for anti-SRBC IgM concentration and absence of statistical significance, and absence of treatment related changes in spleen and thymus weight (see below), the findings are not considered to demonstrate significant suppression of SRBC-specific antibody response, up to and including dose levels that produced general toxicity (i.e. a statistically significant decrease of mean body weight gain at 3000 ppm of 34%).

In contrast, a statistically significant decrease in mean anti-SRBC IgM concentration (93%) was seen with the positive control, which was stated to be within the usual laboratory range observed with cyclophosphamide (Table KIIA 5.10.2/04). Thus, the finding with the positive control, demonstrated the sensitivity of the assay to detect immune-suppressive effects.

Table KIIA 5.10.2/04. SRBC-specific IgM ELISA results on study Day 30

SRBC-specific IgM (U/mL)				
Dose BYI 0260 (ppm)				**Positive control (mg/kg bw/d)**
0	**125**	**600**	**3000**	**3.5**
40516±23875	62920±41649 (55%)	34286±30710 (-15%)	33354±26016 (-18%)	**2732±1737** ** **(-93%)**
Range of individual values within each dose group (U/mL)				
17538 - 84944	17708 - 146500	9092 - 105300	3672 - 90028	554 – 5945

** denotes a value that is statistically significant at the $p<0.01$ level. No other changes were statistically significant (i.e., at least $p\leq0.05$). Data excerpted from pages 27, 72, 101, and 102 of the study report.

2. Gross Pathology

All macroscopic changes seen in BYI 02960 treated animals were considered incidental and not treatment-related. In the positive control (cyclophosphamide) group, atrophic/small spleen and/or thymus was seen in 6/10 and 5/10 females respectively, and correlated with the observed decrease in these organ weights (see below).

3. Organ Weights (spleen and thyroid)

Compared to controls, decreases (not statistically significant) of 2% to 6% in absolute spleen weight were seen in BYI 02960 treated dose groups, with a 6% decrease seen at 3000 ppm. However, a 6% decrease was seen in terminal body weight, and at 3000 ppm a marginal (1%) increase was seen in relative (to body weight) spleen weight. Similarly, while a slight decrease was seen in absolute thyroid weight (4%, not statistically significant) at 3000 ppm, a marginal

increase (2%) was seen in relative (to body weight) thyroid weight at this dose level (**Table KIIA 5.10.2/05**). Thus, no treatment related effect was seen on spleen and thyroid organ weight.

In contrast, for the positive control cyclophosphamide a statistically significant decrease of 34% and 32% was seen for absolute and relative (to body weight) spleen weight respectively, along with a statistically significant decrease of 28% and 27% for absolute and relative (to body weight) thyroid weight respectively (Table KIIA 5.10.2/05).

Table KIIA 5.10.2/05. Spleen and thymus organ weights

Organ	Dose (ppm)				Positive
	0	125	600	3000	control
Spleen abs (g)	0.762±0.122	0.743±0.110 (-2%)	0.732±0.135 (-4%)	0.720+0.096 (-6%)	**0.500±0.049**** **(-34%)**
Spleen RelBW (%)	0.3161±0.0380	0.3115±0.0367 (-2%)	0.3007±0.0391 (-5%)	0.3187±0.0358 (1%)	**0.2136±0.017**** **(-32%)**
Thymus abs (g)	0.544±0.130	0.505±0.075 (-7%)	0.591±0.154 (9%)	0.522±0.104 (-4%)	**0.389±0.063**** **(-28%)**
Thymus RelBW (%)	0.2259±0.0482	0.2126±0.0330 (-6%)	0.2420±0.0532 (8%)	0.2298±0.0364 (2%)	**0.1656±0.0202**** **(-27%)**

** Denotes statistical significance at the p≤0.01 level. Data excerpted from pages 29 and 75 of the study report, except for per cent changes of test substance groups from control, which were calculated by the evaluator using Microsoft Excel and rounded to integers.

The study authors regarded the No Observed Effect Level (NOEL) as 600 ppm (~50 mg/kg bw/d), based on a 6% lower group mean terminal bodyweight at 3000 ppm relative to controls that was accounted for by decreased food consumption. There was no discussion of a No Observed Adverse Effect Level (NOAEL). They also regarded the test substance to not have demonstrated any immunotoxic potential as measured by SRBC-specific IgM production at up to and including the highest dose tested of 3000 ppm (~230 mg/kg bw/d). The evaluator agrees with the study author that under the conditions of this study, there were no indications of test substance immunosuppressive potential up to and including the highest dose tested of 3000 ppm (~230 mg/kg bw/d). The evaluator also considers identification of a NOAEL for systemic toxicity to be inappropriate in this study type, due to the limited range of observations and parameters measured in comparison to a standard 28-day toxicity study.

III. CONCLUSION

Under the conditions of this study, BYI 02960 did not affect mortality, clinical signs, organ weights (i.e. spleen, thymus, and brain), or necropsy findings. Based on the lack of dose dependency and absence of statistical significance of decreases in SRBC-specific IgM at 600 and 3000 ppm, overall there were no indications of test substance immunosuppressive potential up to and including the highest dose tested of 3000 ppm (~230 mg/kg bw/d).

The immunotoxicity NOAEL is the highest dose tested of 3000 ppm (230 mg/kg bw/d), based on an absence of test substance related immunotoxicity findings at this dose.

This study is fully reliable (acceptable/guideline) and satisfies the guideline requirements (OPPTS 870.7800) for an immunotoxicity study in rats.

Primary review: Australia

Secondary review: PMRA & US EPA

Report: Capt A (2009a) BYI 02960. Exploratory 28-day toxicity study in the rat by gavage. Report amendment N°l. Bayer CropScience, 355, rue Dostoievski, BP 153, 06903 Sophia Antipolis Cedex, France. Laboratory Report No**.:** SA 06075. February 24, 2009. Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N°TXRXX297. Bayer CropScience File No. M-283421-02-2. MRID 48844149. PMRA 2239332. Unpublished.

Guidelines: As a preliminary study, it was not designed to meet regulatory requirements.

GLP: No. This study was not conducted in compliance with GLP in that it was not subjected to specific Quality Assurance inspections and dosing suspensions were not analysed for homogeneity, stability and concentration. However, signed and dated statements stating that the study was performed according to standard operating procedures which were previously accepted and periodically inspected by the Quality Assufance unit. A signed Data Confidentiality statement was presented in the report.

Executive summary:

In a 28-day oral gavage study (MRID 48844149), BYI 02960 in corn oil:ethanol:water at 80:10:10 (v/v) vehicle was administered daily to male and female Wistar (Rj:WI [IOPS HAN]) rats (5/sex/dose) at 0, 75, 200, or 350 mg/kg bw/day (final dosing volume of 5 mL/kg bw/day) for either 30 or 31 days. Parameters evaluated included body weight, body weight gain, food consumption, clinical signs, clinical pathology, organ weights, gross and microscopic pathology, and hepatic biochemical measurements of specific cytochrome P-450 enzymatic activities.

At 75 mg/kg bw/day, salivation in all animals on several days from Study Day 13 onwards and prominent lobulation of the liver in a single male rat without any associated microscopic findings were observed. However, these two findings in isolation at this dose level were not considered to be adverse.

At 200 mg/kg bw/day, one female was found dead on study day 2, but the cause of death was not identified. A decrease in food consumption (↓16%) was seen in females during study week 1; changes in clinical chemistry parameter such as decreases in total bilirubin (↓50% in males and ↓56% in females), increased creatinine in females (↑24%), decreased glucose level (↓28%) in males, increased triglyceride level (↑93%) in females, increased ALT (↑30%) in females, and increase liver weight in females (↑25%) were seen. In addition, in females, absolute and relative (relative to body weights and to brain weights) liver weights were increased (↑25% for absolute liver weight, ↑24% relative to body weight, and ↑21% relative to brain weights); enlarged liver and prominent liver lobulation were also present. Histopathology data showed increased incidence of marked diffused centrolubular hepatocellular hypertrophy in males (5/5) and females (3/4). An increase incidence of thyroid follicular cell hypertrophy was found in males

(4/5).

At 350 mg/kg bw/day group, similar adverse effects as those found in 200 mg/kg bw/day group were observed, and most of effects presented greater severity. Two females were found dead with no identifiable cause. Decreased food consumption was seen in both males (↓16%) and females (↓29%); changes in clinical chemistry parameter such as decreases in total bilirubin (↓67% in males and ↓69% in females), decreased glucose level (↓47%) in males and females (↓26%), increased creatinine in females (↑24%), increased triglyceride level in males (↑66%) and in females (↑236%), and increased ALT (↑55%) in females were seen. In addition, in females, absolute and relative (relative to body weights and to brain weights) liver weights were increased (↑49% for absolute liver weight, ↑45% relative to body weight, and ↑46% relative to brain weights); increased incidence of enlarged liver (3/5 in male and 3/3 in females) and prominent liver lobulation (5/5 in males and 2/3 in females) were also present. Histopathology data showed increased incidence of marked diffused centrolubular hepatocellular hypertrophy in males (5/5) and females (3/3). An increase incidence of thyroid follicular cell hypertrophy was found in males (5/5) and females (3/3).

Hepatocellular enzymatic activity assays showed that EROD activity was not clearly affected by treatment. As BROD activity was induced at 350 mg/kg bw/day in both sexes and at 200 mg/kg bw/day in males, with no clear induction of PROD activity, the findings suggest that BYI 02960 is an inducer of the Cytochrome P-450 3A family.

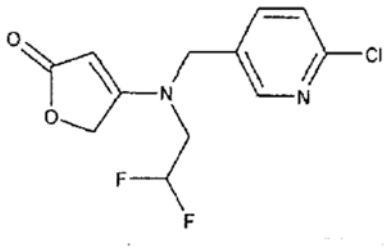
The no-observed-adverse-effect-level (NOAEL) was determined to be 75 mg/kg bw/day; LOAEL was 200 mg/kg bw/day based on decreased mean food consumption in females between Study Day 1 and 8; a decrease in mean total bilirubin in both sexes, a decrease in mean glucose in males, an increase in mean triglycerides and mean creatinine, liver effects [characterized by increases in ALT and ALP, an increase in mean absolute liver weight, mean relative (to body and brain weights) liver weight in both sexes, enlarged and prominent lobulation of the liver in males; minimal to moderate centrilobular hepatocellular hypertrophy of the liver in both sexes], and increased incidence of diffuse follicular cell hypertrophy of the thyroid in males.

This study is considered useful for dose selection and provides information for evaluating the toxicity of this chemical. It is classified as acceptable/non-guideline.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI02960



Lot/Batch #:	NLL 7780-16-5:
Purity:	98.3%
Description:	Orange, amorphous solid
CAS #	Not reported
Stability of test compound:	The test material was stated to be stable for six months if stored as recommended (date of analysis 19 May 2006, storage conditions unspecified).

2. Vehicle and/or positive control: Corn oil:ethanol:water at 80:10:10 (v/v)
3. Test animals

Species:	Rats
Strain:	Wistar (Rj:WI (IOPS HAN))
Age at dosing:	Six weeks old
Weight at dosing:	189 to 208 g (males), 162 to 188 g (females)
Source:	R. Janvier, Le Genest St Isle, France
Acclimation period:	7 days
Diet:	Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum.*
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Individually in suspended, stainless steel, wire-mesh cages.

4. Environmental conditions

Temperature:	20 to 24°C
Humidity:	40 to 70%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Initiated/completed

31-July-2006 to 8-Nov-2006

2. Animal assignment and treatment

The dosing regimen of 0, 75, 200, or 350 mg/kg bw/day was stated to be based on results from a 14-day oral gavage dosing study with BYI 02960 (Capt, 2006; Study No. SA 06074). No further details provided.

Four groups of 5 rats/sex/dose were administered BYI 02960 at 0, 75, 200, or 350 mg/kg bw/d in corn oil:ethanol:water at 80:10:10 (v/v) daily by oral gavage for at least 28 days (either 30 or 31 days) using a nominal dosing volume of 5 mL/kg bw/d (Table 1).

An automatic procedure was used to select animals for the study from the middle of the weight range of the available animals, such that bodyweights were within 20% of the mean bodyweight on the day of randomisation. Animal housing and husbandry were in accordance with the provisions of the *Guide to the Care and Use of Laboratory Animals* (USPHS-NIH Publication No. 86-23) and "Le Guide du Journal Officiel des Communautes Europeennes L358, 18 Decembre 1986, n° 86/609/CEE du 24 Novembre 1986".

Table 1: Study design

Group	No./sex/dose	Dose levels (mg/kg bw/d)	
		M	F
1	5	0	0
2	5	75	75
3	5	200	200
4	5	350	350

Data excerpted from page 18 of the study report.

3. Dose preparation and analysis

The dosing formulations were prepared by suspending appropriate amounts of the test material in corn oil:ethanol:water at 80:10:10 (v/v) to produce the intended dosing concentrations (w/v). Four formulations were prepared for the study at each concentration for both sexes, and they were stored at room temperature when not in use.

The homogeneity and concentrations of the dosing formulations were analysed and stated to be within 91 to 102% (page 25 of the study report), and stability was reported to be demonstrated at 1 and 100 g/L for at least 28 days at room temperature in another study (SA 06225). However, in contrast it is stated in the GLP statement (page 6 of the study report) that dosing suspensions were not analysed for homogenicity, stability and concentration. Thus, it is unclear which statement is correct.

4. Statistics

Statistical analyses of body weight, bodyweight gain, food consumption, absolute and relative organ weight, hematology and clinical chemistry parameters were carried out separately for males and females using non-parametric tests Kruskal-Wallis test followed by Dunn test (2-sided) if significant ($p<0.05$) or using parametric ANOVA followed by Dunnett test (2-sided) if significant ($p<0.05$), as determined to be appropriate by Bartlett's test for homogeneity (non-parametric analyses if significant $p<0.05$ and parametric analysis if not significant).

Additionally, for Body weight and average food consumption/day parameters if Bartlett's test for homogeneity was significant ($p<0.05$) additional parametric analyses (ANOVA followed by Dunnett test (2-sided) if significant ($p<0.05$)) was performed on log transformed data in addition to non-parametric analyses of the raw mean data.

Additionally, for hematology parameters if Bartlett's test for homogeneity was significant ($p<0.05$) additional parametric analyses (ANOVA followed by Dunnett test (2-sided) if significant ($p<0.05$)) was performed on square root transformed data in addition to non-parametric analyses of the raw mean data.

C. METHODS

1. Observations

Animals were observed at least twice daily for mortality and morbidity on weekdays and once daily on weekends. Detailed clinical observations were undertaken weekly and the nature, onset, severity, reversibility, and duration of clinical signs were recorded.

Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose faeces.

2. Body weights

All animals were weighed at study initiation and at necropsy, and on a weekly basis over the study period. Body weight change was calculated for each week and for the total study duration.

3. Food consumption, food efficiency, and daily intake

Food consumption was recorded weekly for each animal. Daily intake was calculated from food consumption and body weight data. Food use efficiency estimates were not undertaken.

4. Clinical pathology (haematology, coagulation, and clinical chemistry)

Blood samples were collected for all animals on study day 30 or 31. The following hematology, clinical chemistry, and coagulation parameters were evaluated on the samples.

<u>Haematology and coagulation</u>

red blood cell count	hemoglobin
reticulocyte count	hematocrit
platelet count	mean corpuscular (cell) volume
white blood cell count	mean corpuscular (cell) hemoglobin
differential white blood cell count	prothrombin time

mean corpuscular (cell) hemoglobin concentration
microscopic blood smear examination

Clinical chemistry parameters

aspartate aminotransferase	total protein
alanine aminotransferase	albumin
alkaline phosphatase	total bilirubin
urea	creatinine
total cholesterol	triglycerides

glucose

5. Urinalysis

Urinalysis was not undertaken.

6. Biochemistry/ mechanistic parameters

A portion of the liver from all surviving animals was homogenised and hepatic microsomes prepared. Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum.

Additionally, specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using the substrates ethoxyresorufin (EROD), pentoxyresorufin (PROD), and benzoxyresorufin (BROD) over a period of 2, 5, or 7 minutes at 37°C, and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid with lauric acid used as substrate.

Microsomes induced by reference compounds β-naphtoflavone, phenobarbital, and clofibric acid served as positive controls to verify the reliability of the assays.

7. Sacrifice and pathology

At termination (study day 30 and 31), animals were sacrificed by exsanguination under deep anesthesia (inhalation of isoflurane) following an overnight fast. Gross examination was performed on all animals. Organs that were weighed are listed in Table 2. Organ weight/final body weight ratios were calculated. Tissues collected from all animals and all gross lesions were processed to slides and evaluated microscopically (Table 2).

Table 2. Organs/tissues collected for pathological examination

Organ	Organ weighed	Collected and preserved	Microscopic/histopathologic evaluation conducted
Brain	X		

Spleen	X	X	X
Liver	X	X	X
Kidneys	X	X	X
Adrenal gland	X	X	X
Thyroid gland	X	X	X
Parathyroid glands	X	X	
Testes	X	X	X
Epididymides	X	X	X
Ovaries	X	X	X
Uterus		X	X
Vagina		X	X
Pituitary	X	X	X
Gross lesions		X	X

Data excerpted from page 220 and 221 of the study report.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity and mortality

Increased salivation was observed in all treated animals (5/5) at all dose levels, but salivation was not seen in any controls. The toxicological significance of salivation as an isolated observation at 75 mg/kg bw/day could not be determined. The US EPA secondary reviewer had analyzed all the relevant studies (acute neurotoxicity study in rats, developmental toxicity in rats, and acute toxicity study in rats) on flupyradifurone to determine if gavage dosing, vehicle, or strain of rats employed would have influenced the observation of salivation at all dose levels. No consistent results was found. Therefore it would not be appropriate to employ salivation as a toxicity endpoint in establishing NOAEL or LOAEL.

At 350 and 200 mg/kg bw/day, it was observed from study day 2 and on most days until the end of the study, while at 75 mg/kg bw/day it was observed on several occasions between study day 13 and 24.

Additionally, tremors were seen in one female at 75 mg/kg bw/day, but based on the absence of this finding at higher dose levels was considered to be unrelated to the test material.

2. Mortality

At 350 mg/kg bw/day, two females were found dead on study day 6, and one female at 200 mg/kg bw/day was found dead on day 2. The only treatment related clinical sign in these animals was increased salivation on/from study day 2. The cause of death of these animals was unknown.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 350 mg/kg bw/day, mean body weight was decreased in males over the study period and at termination (study day 29) was decreased by 6% (not statistically significant compared to controls). In the same animals, a statistically significant 27% decrease in body weight gain was observed from study day 1 to 8 compared to controls. At termination a decrease (not statistically significant) in body weight gain of 12% was seen in males at 350 mg/kg bw/day. Although not statistically significant, the magnitude of the body weight gain decrease still apparent at study termination was considered adverse.

Mean body weight gain was reduced by 17% (not statistically significant) in females at 350 mg/kg bw/day between study days 1 and 8, but thereafter was higher than that seen in controls and a similar decrease was noted in females at 75 mg/kg bw/day. Overall, mean cumulative body weight gain was increased by 24% in females at 350 mg/kg bw/day compared to controls at termination which was not considered to be an adverse finding. No treatment related changes were observed in body weight or body weight gain at 200 and 75 mg/kg bw/day in either sex.

Table 3. Mean body weights and body weight gains

Study day	Body weight (g)							
	Males				Females			
	Dose (mg/kg bw/d)							
	0	**75**	**200**	**350**	**0**	**75**	**200**	**350**
1	198±5	196±8 (-1%)	197±5 (-1%)	195±6 (-2%)	174±3	175±6 (1%)	171±10 (-2%)	171±7 (-2%)
8	257±11	251±12 (-2%)	249±9 (-3%)	238±9 (-7%)	197±7	194±6 (-2%)	197±14 0	189±14 (-4%)
15	309±14	301±18 (-3%)	306±12 (-1%)	284±19 (-8%)	218±12	211±9 (-3%)	219±14 (0%)	222±18 (2%)
22	346±25	336±17 (-3%)	350±19 (1%)	328±24 (-5%)	232±14	225±3 (-3%)	237±16 (2%)	245±9 (6%)
29	378±26	367±16 (-3%)	372±35 (-2%)	355±32 (-6%)	245±13	240±8 (-2%)	248±21 (1%)	258±8 (5%)
Cumulative body weight gain (g)								
8	59±10	55±7 (-7%)	52±7 (-12%)	**43±8 (-27%)***	23±6	19±5 (-17%)	25±3 (9%)	19±6 (-17%)
15	111±14	104±12 (-6%)	109±11 (-2%)	89±19 (-20%)	44±9	37±7 (-16%)	47±4 (7%)	52±11 (18%)
22	149±25	140±13 (-6%)	153±19 (3%)	133±24 (-11%)	58±12	50±6 (-14%)	65±7 (12%)	**75±5 (29%)***
29	180±26	170±12 (-6%)	176±34 (-2%)	159±31 (-12%)	71±12	65±9 (-8%)	76±13 (7%)	88±4 (24%)

* denotes values that are statistically significant at the p≤0.05 level. Values in parentheses represent the percent change from the control mean.
Data excerpted from pages 46 47, 49 and 50 of the study report.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

At 350 mg/kg bw/day, mean daily food consumption was decreased by 17% in males (statistically significant) and by 29 % in females (statistically significant) between Study Day 1 and 8, and by 8% (not statistically significant) in males between Study Day 9 and 15 relative to control group means. Thereafter, mean food consumption was comparable to or slightly higher than the controls.

At 200 mg/kg bw/day, mean food consumption was unaffected by treatment in males. In females, mean food consumption was reduced by 16% (statistically significant) between Study Day 1 and 8 in comparison with the controls. Thereafter, mean food consumption was comparable to or slightly higher than the controls.

At 75 mg/kg/day, mean food consumption was unaffected by treatment in either sex.

Food use efficiency was not determined by the study author.

Table 4. Mean daily food consumption

Study day interval	Mean food consumption/d (g)							
	Males				Females			
	Dose (mg/kg bw/d)							
	0	75	200	350	0	75	200	350
1 to 8	26.5±2.1	24.3±1.6 (-8%)	25.6±1.8 (-3%)	**22±1.6 (-17%)****	19.2±0.9	17.8±0.5 (-7%)	**16.2±2.4 (-16%)***	**13.6±1.6 (-29%)****
9 to 15	27±2.2	25.8±1.9 (-4%)	28.4±2 (5%)	24.9±3 (-8%)	19.3±1.8	18.6±1.1 (-4%)	18.3±1.8 (-5%)	19.2±2.6 (-1%)
16 to 22	27±3.2	24.9±1.4 (-8%)	28.5±3.3 (6%)	27.3±2.3 (1%)	18.9±1.3	19.3±1.2 (2%)	20±1.5 (6%)	21±0.6 (11%)
23 to 29	25.1±3.5	24.0±0.7 (-4%)	26±4.3 (4%)	25.7±3 (2%)	18.8±0.8	20.1±0.5 (7%)	19.9±1.1 (6%)	19.7±0.7 (5%)

* denotes values that are statistically significant at the p≤0.05 level, or ** p<0.01 level.
Data excerpted from pages 55 and 56 of the study report.

D. CLINICAL PATHOLOGY

1. Haematology

There were no treatment related effects on haematology parameters in either sex.

2. Clinical chemistry

Treatment-related and statistically significant changes were observed at 350 and/or 200 mg/kg bw/day and included decreased total bilirubin in both sexes, and increased creatinine and triglyceride concentration, and alanine aminotransferase and alkaline phosphatase activities in females only, and decreased glucose in males. Additionally, although not statistically significant, the magnitude of the increase in mean triglyceride

concentration seen in males at 350 mg/kg bw/day (66%) and in females at 200 mg/kg bw/day (93%), and decrease in glucose in females at 350 mg/kg bw/day, were also considered to be treatment related (Table 5).

In contrast, although a statistically significant decrease was seen in mean total bilirubin in males at 75 mg/kg bw/day (42%), it was not considered to be treatment related as only one animal was affected (with a total bilirubin concentration of 0.0 umol/L, while the mean of the remainder of the group was 0.85 umol/L).

The slightly higher creatinine concentration noted at 200 mg/kg bw/day in males was considered incidental in absence of a similar change being observed at the higher dose level of 350 mg/kg bw/day.

No other treatment-related change was noted for the parameters assayed.

Table 5. Selected clinical chemistry parameters

Parameter	Males				Females			
	Dose (mg/kg bw/d)							
	0	**75**	**200**	**350**	**0**	**75**	**200**	**350**
Total bilirubin (µmol/L)	1.2±0.3	**0.7±0.4 (-42%)***	**0.6±0.2 (-50%)***	**0.4 ±0.2 (-67%)****	1.6±0.4	0.9±0.2 (-44%)	**0.7±0.0 (-56%)***	**0.5±0.1 (-69%)****
Glucose (mmol/L)	5.42±0.69	4.60±0.38 (-15%)	**3.92±0.53 (-28%)****	**2.87±0.37 (-47%)****	4.80±0.43	5.07±0.77 (6%)	5.12±0.93 (7%)	3.57±0.17 (-26%)
Triglycerides (mmol/L)	1.14±0.52	1.5±0.51 (32%)	1.32±0.28 (16%)	1.89±0.84 (66%)	0.45±0.13	0.46±0.14 (2%)	0.87±0.35 (93%)	**1.51±0.56 (236%)***
Creatinine (µmol/L)	50±2	51±2 (2%)	**54±4 (8%)***	53±2 (6%)	51±3	56±1 (10%)	**63±7 (24%)****	**63±9 (24%)***
ALAT (IU/L)	39±6	43±5 (10%)	38±10 (-3%)	46±9 (18%)	33±3	35±4 (6%)	**43±2 (30%)****	**51±3 (55%)****
ALP (IU/L)	165±15	163±36 (-1%)	166±27 (1%)	165±38 (0%)	95±13	110±14 (16%)	96±3 (1%)	**129±26 (36%)***

'*' denotes values that are statistically significant at the p≤0.05 level, or ** p<0.01 level.

Data excerpted from pages 65 and 66 of the study report.

E. BIOCHEMISTRY/MECHANISTIC PARAMETERS

Mean total cytochrome P-450 contents were slightly increased by approximately 1.2 to 1.3-fold relative to controls in males at 75 and 200 mg/kg bw/d respectively, with no significant change seen in males at 350 mg/kg bw/day, and by approximately 1.2-fold in females at both 200 and 350 mg/kg bw/day.

BROD activity was increased at 350 mg/kg/day by approximately 25-fold in males and 28-fold in females. At 200 mg/kg/day, BROD activity was increased by 25-fold in

males and 8-fold in females. The findings at 350 mg/kg bw/day in both sexes and at 200 mg/kg bw/day in males were also comparable to the positive control phenobarbital (BROD increases of 33-fold in males and 63-fold in females). At 75 mg/kg/day, BROD activity was slightly increased by 8-fold in males (Table 6).

EROD activity had a tendency to be very slightly increased at 350 mg/kg/day (3-fold in males and 4-fold in females) and at 200 mg/kg/day (4-fold) in females compared to control values. However, compared to the positive control beta-naphthoflavone (EROD increases of 4-fold in males and 6-fold in females), together with the high inter-individual variability in BYI02960 treated animals at these dose levels, the magnitude of these increases remained at a low level and is not considered to provide a clear relationship to the test material (Table 6).

PROD activity was very slightly increased at 350 mg/kg/day in both sexes (5-fold in males, 4-fold in females) and at 200 mg/kg/day in males (5-fold) compared to control values. However, compared to the positive control phenobarbital (PROD increases of 22-fold in males and 33-fold in females), together with the high inter-individual variability in BYI02960 treated animals at these dose levels, the magnitude of the increase remained at a very low level and is not considered to provide a clear relationship to the test material (Table 6).

The lauric acid hydroxylation levels were not affected by treatment in either sex.

It should be noted, that the observed changes produced by the positive controls were reported from in a separate 28-day oral gavage dosing study in Wistar rats not submitted for this evaluation (Study No. SA 04047).

EROD activity was not found to be clearly affected by treatment. As BROD activity was induced at 350 mg/kg/day in both sexes and at 200 mg/kg/day in males, with no clear induction of PROD activity, the findings suggest that BYI 02960 is an inducer of the Cytochrome P-450 3A family.

Table 6: Hepatotoxicity findings: enzymatic activities

	Male				**Female**			
	Dose (mg/kg bw/d)							
	0	**75**	**200**	**350**	**0**	**75**	**200**	**350**
Total P450 content (nmol/mg prot)	1.41 ±0.19	1.75 ±0.29	1.84 ±0.22	1.54 ±0.14	1.06 ±0.17	1.18 ±0.15	1.25 ±0.23	1.29 ±0.34
BROD (pmol/min/mg prot)	5.18 ±1.84	42.74 ±8.39	128.82 ±41.95	128.36 ±47.24	2.23 ±0.50	7.70 ±4.22	18.91 ±8.30	63.45 ±52.50
EROD (pmol/min/mg prot)	39.40 ±7.15	77.59 ±20.91	62.55 ±9.90	110.12 ±50.05	44.73 ±7.03	86.09 ±22.98	180.06 ±85.96	175.60 ±39.72
PROD (pmol/min/mg prot)	7.12 ±1.34	18.16 ±3.80	37.08 ±7.88	37.01 ±11.50	4.77 ±0.82	8.18 ±3.14	13.44 ±2.76	19.77 ±9.91
Lauric acid (nmol/min/mg prot)	0.90	0.73	1.16	1.11	0.66	0.98	1.29	1.06

Standard deviations are not available for lauric acid hydroxylation.
Statistical analysis was not undertaken. Data excerpted from pages 87 to 96 of the study report.

F. SACRIFICE AND PATHOLOGY

1. Organ weight

Treatment related and statistically significant mean organ weight changes were seen in relative (to body weight) liver weight in males at 350 mg/kg bw/day (23%), relative (to body weight) liver weight in females at 200 and 350 mg/kg bw/day (24% and 45% respectively), and absolute (49%) and relative (to brain weight) liver weight in females at 350 mg/kg bw/day (49% and 46% respectively). At 200 mg/kg bw/day, although not statistically significant the increases seen in absolute and relative (to body weight and brain weight) in males (10%, 11% and 11% consistently), and increases in absolute and relative (to brain weight) in females (25% and 21% respectively) were considered treatment related and adverse, as were the increases in absolute and relative (to brain weight) liver weight in males at 350 mg/kg bw/day (14% and 15% respectively). While increases in liver weights (absolute and relative to body weight and brain weight, which were not statistically significant) were seen at 75 mg/kg bw/day in both sexes, changes of 10% or greater were not consistently seen and the observed liver findings (and also were not associated with treatment related histopathological changes) are not considered adverse (Table 7).

Non-statistically significant increases were also seen in other organs, such as the thyroid and kidney. However, increases were either not consistently seen across the absolute and relative (to body weight and brain weight) values and/or were seen in the absence of a dose response relationship and were considered incidental and not related to treatment (Table 7).

Non-statistically significant decreases in spleen weight (absolute and relative to body weight and brain weight) were seen in males at 200 and 350 mg/kg bw/day and in females at 350 mg/kg bw/day (Table 7). These changes were not associated with any treatment-related pathological findings.

Table 7. Selected organ weight findings

Parameter	Males				Females			
	Dose (mg/kg bw/d)							
	0	75	200	350	0	75	200	350
Terminal BW (g)	352.4±25.1	342.5±13.3 (-3%)	347.8±28.8 (-1%)	326.1±30.8 (-7%)	229.8±13.2	223.5±4.4 (-3%)	231.4±20.3 (1%)	236.3±6.7 (3%)
Liver Abs (g)	10.92±1.68	9.97±0.76 (-9%)	12.01±1.72 (10%)	12.43±1.12 (14%)	5.86±0.32	6.37±0.15 (9%)	7.32±0.82 (25%)	**8.74±0.27 (49%)***
Liver relBW	3.095±0.373	2.913±0.101 (-6%)	3.442±0.279 (11%)	**3.814±0.08 (23%)***	2.552±0.065	2.85±0.016 (12%)	**3.157±0.132 (24%)***	**3.704±0.22 (45%)****
Liver relBrain	542.70±88.466	482.53±30.783 (-11%)	602.57±74.514 (11%)	625.67±52.142 (15%)	315.09±14.599	328.83±7.351 (4%)	382.27±40.134 (21%)	**460.55±33.184 (46%)****
Thyroid Abs (g)	0.0197±0.0038	0.0232±0.0041 (18%)	0.0207±0.0038 (5%)	0.0232±0.0032 (18%)	0.0168±0.0027	0.0202±0.0023 (20%)	0.018±0.0025 (7%)	0.0203±0.0029 (21%)
Thyroid relBW	0.0056±0.0011	0.00675±0.001 (21%)	0.00594±0.001 (6%)	0.00716±0.00121 (28%)	0.00733±0.00117	0.00902±0.00106 (23%)	0.00788±0.00165 (8%)	0.00859±0.00104 (17%)
Thyroid relBrain	0.9741±0.17097	1.1340±0.1829 (16%)	1.0347±0.16403 (6%)	1.1673±0.16081 (20%)	0.90197±0.1177	1.04103±0.1264 (15%)	0.94461±0.1468 (5%)	1.07121±0.1707 (19%)
KidneyAbs (g)	2.45±0.19	2.36±0.18 (-4%)	2.45±0.17 (0%)	2.36±0.23 (-4%)	1.63±0.2	1.69±0.15 (4%)	1.77±0.13 (9%)	1.77±0.26 (9%)
Kidney relBW	0.695±0.043	0.689±0.033 (-1%)	0.711±0.099 (2%)	0.724±0.05 (4%)	0.707±0.052	0.757±0.06 (7%)	0.766±0.033 (8%)	0.751±0.119 (6%)
Kidney relBrain	121.42±9.116	115.74±9.485 (-5%)	123.66±12.504 (2%)	118.57±10.776 (-2%)	87.48±9.997	87.38±7.342 (0%)	92.55±6.808 (6%)	93.00±10.87 (6%)
Spleen Abs (g)	0.933±0.172	0.875±0.179 (-6%)	0.765±0.088 (-18%)	0.682±0.107 (-27%)	0.632±0.098	0.664±0.084 (5%)	0.619±0.042 (-2%)	0.512±0.026 (-19%)
Spleen relBW	0.264±0.036	0.255±0.045 (-3%)	0.221±0.027 (-16%)	0.209±0.025 (-21%)	0.275±0.043	0.297±0.034 (8%)	0.269±0.028 (-2%)	0.217±0.014 (-21%)
Spleen relBrain	46.388±9.338	42.757±8.003 (-8%)	38.434±3.988 (-17%)	34.315±5.065 (-26%)	33.934±5.302	34.204±3.759 (1%)	32.343±1.828 (-5%)	26.996±2.937 (-20%)

* denotes values that are statistically significant at the p≤0.05 level, or ** p<0.01 level. Values in parentheses represent the percent change from the control mean.
Data excerpted from pages 68 to 73 of the study report

2. Gross pathology and histopathology

No treatment related gross findings were observed in the two females and one female that died prior to scheduled sacrifice at 350 and 200 mg/kg bw/day, respectively.

At termination, a dose related increase was seen in enlarged livers in males at 200 mg/kg bw/day and greater, and was also seen in females at 350 mg/kg bw/day. Additionally, a dose related increase was seen in prominent lobulation of the liver in males at 75 mg/kg bw/day and greater, and was also seen in females at 350 mg/kg bw/day. Both findings were absent from concurrent controls (Table 8).

The singular finding of liver lobulation in one male at 75 mg/kg bw/day was not associated with treatment related clinical chemistry changes, liver weight changes or histopathological changes. Consequently, this finding in one male at 75 mg/kg bw/day while potentially treatment related was not considered adverse.

Other macroscopic changes were considered incidental and not treatment related.

Table 8. Summary of gross liver findings

	Males				**Females**			
	Dose (mg/kg bw/d)							
	0	**75**	**200**	**350**	**0**	**75**	**200**	**350**
Enlarged	0/5	0/5	1/5	3/5	0/5	0/5	0/4	3/3
Prominent lobulation	0/5	1/5	2/5	5/5	0/5	0/5	0/4	2/3

Data excerpted from page 28 of the study report.

Treatment related minimal to marked diffuse centrilobular hepatocellular hypertrophy of the liver was seen in all animals (except one female at 200 mg/kg bw/day) at 350 and 200 mg/kg bw/day that demonstrated dose-dependent increases in severity. Additionally, treatment related diffuse follicular cell hypertrophy of the thyroid of minimal to slight severity was seen in all males and 3/5 females at 350 mg/kg bw/day and 4/5 males at 200 mg/kg bw/day (Table 9).

No other treatment related histopathological changes were seen in either sex.

Table 9. Selected histopathological findings

	Males				Females			
	Dose (mg/kg bw/d)							
	0	**75**	**200**	**350**	**0**	**75**	**200**	**350**
Number examined	5	5	5	5	5	5	4	3
Liver - Diffuse centrilobular hepatocellular hypertrophy								
Minimal	-	-	-	-	-	-	2	-
Slight	-	-	4	1	-	-	1	2
Moderate	-	-	1	3	-	-	-	1
Marked	-	-	-	1	-	-	-	-
Total	**0**	**0**	**5 (100%)**	**5 (100%)**	**0**	**0**	**3 (75%)**	**3 (100%)**
Thyroid - Diffuse follicular cell hypertrophy								
Minimal	-	-	4	4	-	-	-	2
Slight	-	-	-	1	-	-	-	1
Total	**0**	**0**	**4 (80%)**	**5 (100%)**	**0**	**0**	**0**	**3 (100%)**

Data excerpted from pages 29 and 30 of the study report.

III. CONCLUSION

The study author determined the no-observed-adverse-effect-level (NOAEL) in both sexes to be 75 mg/kg bw/day. The only findings at this dose level were increased salivation in all animals on several days from Study Day 13 onwards and prominent lobulation of the liver in one male without associated microscopic findings. In the absence of any other changes, these observations were not considered as adverse.

The evaluator agrees with the identified NOAEL in males and females, and agreed with the study author's interpretation of the study findings.

The evaluator determined the NOAEL in both sexes to be 75 mg/kg bw/day; LOAEL was 200 mg/kg bw/day, based on decreased mean food consumption (↓16%) in females between Study Day 1 and 8; a decrease in mean total bilirubin in both sexes (↓50-56%), a decrease in mean glucose in males (↓28%), an increase in mean triglyceride in females (↑93%), an increase in mean creatinine in females (↑24%) and liver effects (characterized by an increase in mean alanine aminotransferase activity in females (↑30%) with associated increase in mean absolute liver weight (↑10-25%), mean relative (to body weight) liver weight (↑11-24%) and mean relative (to brain weight) liver weight (↑11-21%) in both sexes; enlarged and prominent lobulation of the liver in males; minimal to moderate centrilobular hepatocellular hypertrophy of the liver in both sexes) and minimal diffuse follicular cell hypertrophy of the thyroid in males.

In additionally at 200 mg/kg bw/day, hepatocellular enzymatic activity assays showed an induction of BROD activity in males with no clear induction of PROD activity that suggested BYI 02960 is an inducer of Cytochrome P-450 3A family.

This study is considered useful for dose selection and provides information for evaluating the toxicity of this chemical. It is classified as acceptable/non-guideline.

Primary review: Australia
Secondary review: PMRA & US EPA

Report: Blanck M (2008) BYI 02960 - Exploratory 28-day toxicity study in the rat by dietary administration. Bayer CropScience, 355, rue Dostoievski, BP 153, 06903 Sophia Antipolis Cedex, France. Laboratory Report No**.:** SA 07047. 1-February-2008. Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N° TXRVP005. Bayer CropScience File No. M-297120-01-2. MRID 48844150. PMRA 2239311. Unpublished.

Guidelines: As a preliminary study, it was not designed to meet regulatory requirements.

GLP: No. This study was not conduced in compliance with GLP in that it was not subjected to specific Quality Assurance inspections and dosing suspensions were not analysed for homogeneity, stability and concentration. However, signed and dated statements stating that the study was performed according to standard operating procedures which were previously accepted and periodically inspected by the Quality Assurance unit. A signed Data Confidentiality statement was presented in the report.

Executive summary

In a 28-day dietary study (MRID 48844150), BYI 02960 was administered daily in the diet to groups of male Wistar (Rj:WI (IOPS HAN)) (5 male rats/dose) at concentrations of 0, 500 or 5000 ppm (0, 33.6 [adjusted to account for a likely 18% degradation of the test material at this dose level] and 385 mg/kg bw/day). Parameters evaluated included body weight, bodyweight gain, food consumption, clinical signs, clinical chemistry (including thyroid hormone analysis), organ weights, gross and microscopic pathology, and hepatic biochemical measurements of specific cytochrome P-450 enzymatic activities.

There were no unscheduled deaths or clinical signs of toxicity in the treated groups.

At 5000 ppm:

Mean body weight was decreased throughout the study and at termination was reduced by 18% (not statistically significant), and a statistically significant decrease of 38% was seen in the overall mean cumulative body weight gain. A statistically significant decrease was seen in food consumption weekly over the study period, being 12% at termination. Statistically significant decreases were seen in mean glucose (46%) and total bilirubin (73%), along with statistically significant increases in urea nitrogen (37%) and total cholesterol (41%). Additionally, an increase of 80% in TSH levels along with and a 19% decrease in mean T4 levels were found. Although neither of these findings was statistically significant, they were considered likely treatment-related. A statistically significant increase in mean relative (to body weight) liver weight (42%) was present, and although not statistically significant the magnitude of the observed increase in absolute liver weight (15%) was also considered treatment related and adverse. A statistically

significant increase was seen in mean relative (to body weight) thyroid weight (32%) and a slight, non-statistically significant increase was seen in absolute thyroid weight (6%), which were associated with histopathological changes. At necropsy, increased incidence of prominent lobulation of the liver (4/5) was seen. Treatment related microscopic changes characterized by slight to moderate diffuse centrilobular hepatocellular hypertrophy were seen in the liver. An increase in the incidence of slight diffuse thyroid follicular cell hypertrophy (5/5) was also seen.

No changes in total P-450 content, mean EROD activity, or mean PROD activities were observed, while at 5000 ppm only a 3.1-fold increase in BROD activity and 2.1-fold increase in UDPGT activity were observed. Furthermore, the thyroid histopathology findings at 5000 ppm could be related to the observations of increased liver enzyme activity as suggested by the increases in UDPGT activity and TSH levels and decreased T4 levels at this dose level.

At 500 pppm:

No test-material-related effects were seen on body weight, body weight gain or food consumption. No toxicologically significant changes in clinical chemistry parameters were seen.. Lobulation of the liver was seen in 2/5 males, but no correlated changes clinical chemistry parameter, or histopathology changes were observed. Therefore, the liver lobulation was not considered adverse.

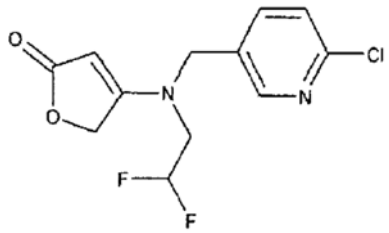
The NOAEL in male rats was determined to be 500 ppm (33.6 mg/kg bw/day). LOAEL was 5000 ppm (385 mg/kg bw/day) based on: decreased body weight, body weight gain and food consumption; decreased glucose and total bilirubin and increased urea nitrogen and total cholesterol; increased absolute and relative (to body weight) liver weight and increased absolute and relative (to body weight) thyroid weight; prominent liver lobulation; and slight to moderate diffuse centrilobular hepatocellular hypertrophy in the liver, and minimal to slight diffuse follicular cell hypertrophy in the thyroid.

This study is considered useful for dose selection and provides information for evaluating the toxicity of this chemical. It is classified as **acceptable/non-guideline** (only male rats were tested).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960



Lot/Batch #:	NLL 7780-27-1
Purity:	99.7%
Description:	Biege powder
CAS #	Not reported
Stability of test compound:	The test material was stated to be stable for one year if stored at room temperature (date of analysis 14-June-2007)

2. Vehicle and/or positive control: Untreated diet

3. Test animals

Species:	Male rats
Strain:	Wistar (Rj:WI (IOPS HAN))
Age at dosing:	At least six weeks old (males)
Weight at dosing:	201 to 214 g (males)
Source:	R. Janvier, Le Genest St Isle, France
Acclimation period:	7 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum.*
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Individually in suspended, stainless steel, wire-mesh cages.

4. Environmental conditions

Temperature:	20 to 24°C
Humidity:	40 to 70%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Initiated/completed

01 June 2007 to 18 October 2007

2. Animal assignment and treatment

The dosing regimen of 0, 500, or 5000 ppm was based on results from the previous rat 28-day oral gavage study (Capt, 2009a, Study No. SA 06075).

Three groups of 5 male rats/dose were administered BYI 02960 at 0, 500, or 5000 ppm in feed daily for at least 28 days, and mean doses were determined to be 0, 40.97, and 385.0 mg/kg bw/day in males respectively (Table 1).

An automatic procedure was used to select animals for the study from the middle of the weight range of the available animals, such that bodyweights were within 20% of the mean bodyweight on the day of randomisation. Animal housing and husbandry were in accordance with the provisions of the *Guide to the Care and Use of Laboratory Animals* (USPHS-NIH Publication No. 86-23) and "Le Guide du Journal Officiel des Communautes Europeennes L358, 18 Decembre 1986, n° 86/609/CEE du 24 Novembre 1986".

Table 1: Study design

Group	No./males/dose	Nominal dietary dose (ppm)	Achieved mean dose level (mg/kg bw/d)
1	5	0	0
2	5	500	40.97*
3	5	5000	385.0

* Adjusted to 33.6 mg/kg bw/d by the study authors to account for a likely 18% degradation of the test material.

3. Diet preparation and analysis

The required amount of the test material was incorporated into the diet by dry mixing to achieve the nominal dietary concentrations at each dosing level. There was one preparation for each concentration. When not in use, prepared diet formulations were stored at approximately -18°C.

The stability of BYI 02960 in the diet was evaluated on a pre-test mix at 50, 500, 5000 and 20000 ppm following storage for at least 28 days (study SA 07131). In the stability study, the freezing and thawing of the diet led to problems in recovery of the compound at the low dose levels (50 and 500 ppm). Recovery rates were between 80% and 87% (18 days frozen + 10 days at room temperature and 34 days frozen + 4 days at room temperature, respectively) and above 90% at 5000 ppm analysed under the same conditions.

The stability of BYI 02960 at 500 ppm was shown to be at 82% of the nominal concentration after 34 days frozen storage and 8 days at room temperature. This degradation rate was taken into account to calculate the achieved dosage in the current study.

Homogeneity analysis of the test diets prepared to the current study found ranges of 97 to 99% of nominal concentrations, which the laboratory regarded as acceptable.

4. Statistics

Statistical analyses of body weight, body weight gain, average food consumption, hormonal, absolute and relative organ weight and clinical chemistry parameters were carried out using the non-parametric Kruskal-Wallis test followed by Dunn test (2-sided) if significant ($p<0.05$) or using parametric ANOVA followed by Dunnett test (2-sided) if significant ($p<0.05$), as determined to be appropriate by Bartlett's test for homogeneity (non-parametric analyses if significant $p<0.05$ and parametric analysis if not significant).

Additionally, for body weight, average food consumption/day and hormonal parameters if Bartlett's test for homogeneity was significant ($p<0.05$) additional parametric analyses (ANOVA followed by Dunnett test (2-sided) if significant ($p<0.05$)) was performed on log transformed data in addition to non-parametric analyses of the raw mean data.

C. METHODS

1. Observations

Animals were observed at least twice daily for mortality and morbidity on weekdays and once daily on weekends. Detailed clinical observations were undertaken weekly and the nature, onset, severity, reversibility, and duration of clinical signs were recorded.

Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose faeces.

2. Body weights

All animals were weighed at study initiation and at necropsy, and on a weekly basis over the study period. Body weight change was calculated for each week and for the total study duration.

3. Food consumption, food efficiency, and daily intake

Food consumption was recorded weekly for each animal. Daily intake was calculated from food consumption and body weight data. Food use efficiency estimates were not undertaken.

4. Clinical pathology (clinical chemistry and hormone analyses)

Haematological and coagulation analysis was not undertaken.

Blood samples were collected following an overnight fast for all animals on study day 30 by puncture of the retro-orbital venous plexus after animals were anesthetized by inhaling isoflurane. The following clinical chemistry and hormone parameters were evaluated on the samples.

Clinical chemistry parameters

aspartate aminotransferase	total protein
alanine aminotransferase	albumin
alkaline phosphatase	total bilirubin
urea nitrogen	creatinine
total cholesterol	triglycerides
glucose	

Hormone parameters

T4 and TSH levels were measured in plasma samples by radio-immunoassay.

5. Urinalyses

Urinalyses were not undertaken.

6. Biochemistry/ mechanistic parameters

A portion of the liver from all surviving animals was homogenised and hepatic microsomes prepared. Samples were stored at below -70°C instead of being processed on the day of necropsy (due to the breakdown of the ultra-centrifuge). This may have led to a denaturation of a part of the cytochrome P-450 contents and its transformation into non-active cytochrome P-420. For this reason, the hepatotoxicity may be under estimated.

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum.

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using the substrates ethoxyresorufin (EROD), pentoxyresorufin (PROD), and benzoxyresorufin (BROD) over a period of 2, 5, or 7 minutes at 37ºC.

Additionally, Phase II enzymatic activity was determined by measuring UDP-glucuronosyltransferase (UDPGT) activity with 4-nitrophenol as the substrate for 3 minutes at 30ºC.

Microsomes induced by reference compounds β-naphtoflavone and phenobarbital served as positive controls to verify the reliability of the assay.

7. Sacrifice and pathology

At termination (study day 30), animals were sacrificed by exsanguination under deep anesthesia (inhalation of isoflurane) following an overnight fast. Gross examination was performed on all animals. Organs that were weighed are listed in Table 2. Organ weight/final body weight ratios were calculated. Tissues collected from all animals and all gross lesions were processed to slides and evaluated microscopically (Table 2).

Table 2. Organs/tissues collected for pathological examination

Organ	Organs weighed	Collected and preserved	Microscopic/histopathologic evaluation conducted
Liver	X	X	X
Kidneys	X	X	X
Thyroid gland (with parathyroid)	X	X	X (except parathyroid)
Testes	X	X	X
Gross observations		X	X

Data excerpted from page 133 of the study report.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

   No clinical signs of systemic toxicity were observed

2. Mortality

   There were no unscheduled mortalities.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 5000 ppm in comparison to controls, mean body weight was decreased by between 17 and 19% throughout the study and was statistically significant for the first 3 weeks of the study (Table 3). At termination, mean body weight was reduced by 18% (not statistically significant) compared to controls. Additionally, mean body weight gain per day was decreased (statistically significant) between Study Day 1 and 8 (approximately 1 g/day compared to 7 g/day in controls) and between Study Day 8 and 15 (approximately 6 g/day compared to 8 g/day in controls). Thereafter, mean body weight gain was similar to the controls. However, compared to controls a statistically significant decrease of 38% (Table 3) was seen in the overall mean cumulative body weight gain (i.e. from study day 1 to day 29).

At 500 ppm, there were no test material related effects on body weight or body weight gain.

Table 3. Mean body weights and body weight gains

Study day	Body weight (g)		
	Dose (ppm)		
	0	500	5000
1	208±4	209±5 (0%)	207±5
8	259±10	260±5 (0%)	214±7 (-17%)**
15	319±13	318±6 (0%)	257±9 (-19%)**
22	365±13	364±4 (0%)	296±13 (-19%)**
29	395±18	403±4 (2%)	323±15 (-18%)
Cumulative body weight gain (g)			
8	51±7	52±2 (2%)	7±2 (-86%)*
15	110±10	109±6 (-1%)	50±6 (-55%)**
22	156±12	155±2 (-1%)	88±12 (-44%)*
29	186±16	194±6 (4%)	116±17 (-38%)**

* denotes values that are statistically significant at the p≤0.05 level, or ** p<0.01 level. Values in parentheses represent the percent change from the control mean.
Data excerpted from pages 35 and 39 of the study report.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

At 5000 ppm compared to controls, a statistically significant decrease was seen in food consumption weekly over the study period, being 39% lower than control in week one and 12% lower than controls at termination (data not shown).

At 500 ppm, there were no test material related effects on food consumption.

Food use efficiency was not determined by the study author.

D. CLINICAL PATHOLOGY

1. Clinical chemistry

At 5000 ppm at termination (Table 4), compared to controls treatment related and statistically significant decreases were seen in mean glucose (46%) and total bilirubin (73%), along with statistically significant increases in urea nitrogen (37%) and total cholesterol (41%).

At 500 ppm at termination, no statistically significant changes were seen in clinical chemistry parameters and those changes that were seen were generally small in magnitude and not considered toxicologically significant (Table 4).

Table 4. Selected clinical chemistry parameters

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

*: Statistically significant from control at: p≤0.05.
** : p≤0.01. Values in parentheses represent the percent change from the control mean.
Data excerpted from page 21 of the study report.

2. Hormone analysis

At 5000 ppm at termination, compared to controls an increase of 81% in mean TSH levels and a 19% decrease in mean T4 levels was seen, neither of which was statistically significant. Although not statistically significant, these findings were considered likely treatment related with the decreased T4 level considered likely interrelated to the increased TSH level, while it was also noted that these findings were associated with a slight increase in UDPGT activity at this dose level (see Sub-section E below).

At 500 ppm compared to controls, the slight increases seen in TSH (16%) and T4 (21%) levels were not considered to be toxicologically significant (Table 5), and it is noted were not associated with an increase in UDPGT activity at this dose level.

Table 5. Hormone parameters

Parameter	Dose (ppm)		
	0	500	5000
T4 (nmol/L)	48.5±9.2	58.7±10.2 (21%)	39.5±6.6 (-19%)
TSH (ng/ml)	9.64+4.77	11.23±5.41 (16%)	17.46±5.38 (80%)

No changes were statistically significant from control at p≤0.05.
Data excerpted from page 22 of the study report.

E. BIOCHEMISTRY/MECHANISTIC PARAMETERS

Due to a breakdown of the ultra-centrifuge at terminal sacrifice, livers were weighed, frozen

and stored at <-70°C instead of being processed on the day of necropsy. This may have led to denaturation of cytochrome P-450 contents and its transformation into non-active cytochrome P-420, and lead to an underestimation of the measured hepatic enzyme activities.

There was no test material related effect on mean total cytochrome P-450 content, mean EROD activity, or mean PROD activity at either dose level. At 5000 ppm, there was an approximate 3.1-fold increase in mean BROD activity and an approximate 2.1-fold increase in mean UDPGT activity (Table 6). Although, BROD activity appeared to be only weakly induced, it is reasonable to assume that it was likely underestimated due to the technical problem (i.e. hepatic microsomes being frozen instead of processed on the day of necropsy) that could have induced the presence of inactive cytochrome P-420 in the microsomes.

Table 6. Selected hepatic enzyme parameters

Parameter	Dose (ppm)		
	0	500	5000
Total P450 content (nmol/mg prot)	1.04+0.19	1.01+0.10	1.01+0.06
EROD (pmol/min/mg prot)	66.58+12.50	72.73+25.78	48.86+7.51
PROD (pmol/min/mg prot)	9.91+0.98	10.46+0.99	14.75+3.32
BROD (pmol/min/mg prot)	15.06+2.03	15.88+5.50	46.73+16.07
UDPGT (nmol/min/mg prot)	12.25+2.06	12.38+1.59	25.8+1.38

No changes were statistically significant from control at $p \leq 0.05$
Data excerpted from page 58 to 66 of the study report.

Overall, these data suggest that BYI 02960 at 5000 ppm (385 mg/kg bw/day) in male rats has the ability to induce the cytochrome P-450 3A family. This finding is consistent with the finding in the previous rat oral gavage study with BYI 02960 (Capt, 2009a; Study No. SA 06075) that also suggested that BYI 02960 is an inducer of the Cytochrome P-450 3A family in male rats at 200 and 350 mg/kg bw/day and in female rats at 350 mg/kg bw/day.

Furthermore, the thyroid findings at 5000 ppm could be related to the observations recorded in the liver, particularly the increase in UDPGT activity at this dose level, as this enzyme represents the major elimination pathway for thyroid hormones. This hypothesis is also supported by the observation of increased TSH and lower T4 levels at 5000 ppm.

F. SACRIFICE AND PATHOLOGY

1. Organ weight

At 5000 ppm, a statistically significant increase was seen in mean liver relative (to body weight) liver weight (42%), and although not statistically significant the magnitude of the observed increase in absolute liver weight at 5000 ppm (15%) was also considered treatment related and adverse. Additionally at 5000 ppm, a statistically significant increase was seen in mean relative (to body weight) thyroid weight (32%), though the absolute liver finding was slight (6%) and not statistically significant. However, histopathological changes to the thyroid were seen at 5000 ppm, and the thyroid organ

weight changes are considered treatment related and adverse.

The slight changes seen in absolute and relative (to body weight) liver and thyroid weight at 500 ppm (not statistically significant) were considered likely incidental and not treatment related (Table 7).

A statistically significant decrease in absolute testis weight was noted at 5000 ppm, which was accompanied by a statistically significant increase in relative (to body weight) testis weight. In the absence of any associated pathology, these changes are considered to be secondary to the body weight effects noted at this dose level.

Table 7. Selected organ weight parameters

Parameter	Dose (ppm)		
	0	**500**	**5000**
Terminal bw (g)	355.7±16.7	361.3±0.7 (2%)	288.0±11.5 (-19%)
Absolute liver weight (g)	9.79±0.70	10.38±0.26 (6%)	11.25±1.20 (15%)
Liver relative to bw (%)	2.751±0.127	2.873±0.070 (4%)	**3.906±0.405 (42%)****
Absolute thyroid weight (g)	0.0171±0.0012	0.0165±0.0016 (-4%)	0.0182±0.0005 (6%)
Thyroid relative to bw (%)	0.00480±0.00022	0.00457±0.00044 (-5%)	**0.00632±0.00017 (32%)****
Absolute testis weight (g)	3.48±0.09	3.25±0.27 (-7%)	3.19±0.13 (-8%)*
Testis relative to bw (%)	0.980±0.037	0.898±0.077 (-8%)	1.110±0.081 (13%)*

* Statistically significantly different from control at p≤0.05.
**Statistically significantly different from control at p≤0.01
Data excerpted from pages 22, 23 and 52 of the study report.

2. Gross pathology and histopathology

At necropsy, prominent lobulation of the liver was seen in 2/5 males at 500 ppm, and 4/5 males at 5000 ppm, and was absent in control animals. All other macroscopic changes were considered incidental and not treatment related.

Treatment related microscopic changes were seen in the liver and thyroid at 5000 ppm. Slight to moderate diffuse centrilobular hepatocellular hypertrophy in the liver, and minimal to slight diffuse follicular cell hypertrophy in the thyroid, was seen in all animals. These findings were absent in animals at 500 ppm and control animals. All other microscopic changes were considered incidental and not treatment related.

The gross macroscopic finding of prominent lobulation of the liver in two males at 500 ppm were not associated with microscopic changes, and while considered treatment related was not considered adverse.

III. CONCLUSION

In this study, only 5 male Wistar (Rj:WI (IOPS HAN)) were treated with 500 or 5000 ppm flupyradifurone (BYI 02960) for 28 days. The study also included analyses of T_4 and TSH levels; the hepatic enzyme activity levels (EROD, PROD, BROD, and UDPGT levels) were also measured. The following results were obtained.

At 5000 ppm:
Mean body weight was reduced by between 17% and 19% throughout the study period (the effect being statistically significant for the first 3 weeks of the study; $p \leq 0.01$) in comparison with the controls. Mean body weight gain per day was markedly reduced between Study Day 1 and 8 (1 g/day compared to 7 g/day in the control; $p<0.01$) and slightly between Study Day 8 and 15 (6 g/day compared to 8 g/day in the control). Thereafter, mean body weight gain per day was similar to the control group. Overall, mean cumulative body weight gain was reduced by 38% compared to the controls by Study Day 29. Mean food consumption was consistently reduced by between 12 and 39% ($p \leq 0.01$) for each measured interval, the effect being more pronounced between Study Day 1 and 8. Statistically significant lower glucose (- 46%; $p \leq 0.01$) and total bilirubin (– 73%; $p \leq 0.05$) concentrations and higher urea (+ 37%; $p \leq 0.01$) and total cholesterol (+ 41%; $p \leq 0.05$) concentrations were observed at the end of the study.

At final sacrifice, mean terminal body weight was 19% lower (not statistically significant) when compared to controls. The mean relative liver weight was 42% higher ($p \leq 0.01$) and mean relative thyroid weights was 32% higher ($p \leq 0.01$) compared to the controls. At the macroscopic examination, prominent lobulation in liver was observed in 4/5 animals. At the microscopic examination, slight to moderate diffuse centrilobular hepatocellular hypertrophy in the liver was observed in all 5000 ppm animals. Minimal to slight diffuse follicular cell hypertrophy was observed in the thyroid gland of all animals.

The assessment of the P-450 isoenzyme activities at the end of the 28-day treatment period revealed increases in mean BROD and mean UDPGT activities with no apparent changes in total cytochrome P-450 contents, mean EROD and PROD activities. Although BROD activity appeared to be only weakly induced, it is likely to have been underestimated due to a technical problem that could have induced the presence of inactive cytochrome P-420 in the microsomes. Overall, these data indicate that BYI 02960 (at this dose level) has the ability to induce the cytochrome P-450 3A family.

At 5000 ppm, hormone analysis showed an increase in TSH (+ 80%) and a slight decrease in T4 by 19% (neither being statistically significant) in the plasma. The histopathological changes in the thyroid at 5000 ppm could be related to the increased liver enzyme activity (supported by the finding of increases in UDPGT activity and TSH levels and decreased T4 levels at this dose level).

At 500 ppm:

No test material related effects were seen on body weight, body weight gain or food consumption Lobulation of the liver in 2/5 males was found, but in the absence of any associated clinical chemistry and histopathological changes this finding was not considered to be adverse.

Based on the results of this study, the NOAEL was 500 ppm (33.6 mg/kg bw/day); LOAEL was 5000 ppm (385 mg/kg bw/day), based on: decreased body weight, body weight gain and food consumption; decreased glucose and total bilirubin and increased urea nitrogen and total cholesterol; increased absolute and relative (to body weight) liver weight and increased absolute and relative (to body weight) thyroid weight; prominent liver lobulation; and slight to moderate diffuse centrilobular hepatocellular hypertrophy in the liver, and minimal to slight diffuse follicular cell hypertrophy in the thyroid.

This study is considered useful for dose selection and provides information for evaluating the toxicity of this chemical. It is classified as acceptable/non-guideline (only male rats were used).

Primary review: Australia
Secondary review: PMRA & US EPA

Report: Blanck O (2007) BYI 02960. Preliminary 28-day toxicity study in the mouse by dietary administration. Bayer CropScience, 355, rue Dostoievski, BP 153, 06903 Sophia Antipolis Cedex, France. Laboratory Report No.**:** SA 07013. 23 November, 2007. Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N° TXRVP001. Bayer CropScience File No. M-294820-01-2. MRID 48844151. PMRA 2239302. Unpublished.

Guidelines: As a preliminary study, it was not designed to meet regulatory requirements.

GLP: No. This study was not conduced in compliance with GLP in that it was not subjected to specific Quality Assurance inspections and dosing suspensions were not analysed for homogeneity, stability and concentration. However, signed and dated statements stated that the study was performed according to standard operating procedures which were previously accepted and periodically inspected by the Quality Assurance unit.A signed Data Confidentiality statement was presented in the report.

Executive summary:

In a 28-day dietary study (MRID 48844151), BYI 02960 was administered daily in the diet to male and female C57BL/6J mice in groups of 5 mice/sex/dose at dietary concentrations of 0, 300, 600, or 1200 ppm (males: 0, 40, 78 and 166 mg/kg bw/day, females 0, 47, 98 and 192 mg/kg bw/day following adjustment to take into account instability of the test material). Parameters evaluated included body weight, body weight gain, food consumption, clinical signs, clinical chemistry, organ weights, and gross and microscopic pathology.

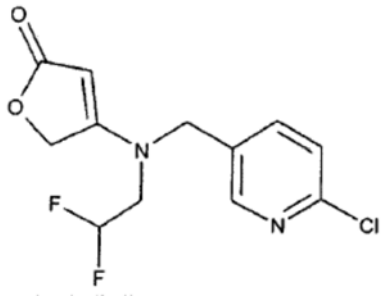
There were no test material related deaths or clinical signs, or changes in body weight, food consumption, organ weight, or gross or microscopic findings in either sex. Additionally, there was no adverse effect on body weight gain in females or clinical chemistry parameters in males. Therefore, there were no adverse effects found on any of the parameters examined in this study. The NOAEL was 1200 ppm (166/192 mg/kg bw/day, M/F) (highest dose tested), and no LOAEL was established.

This study is considered useful for dose selection and provides information for evaluating the toxicity of this chemical. It is classified as acceptable/non-guideline.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960



Lot/Batch #:	NLL 7780-27-1
Purity:	99.7% (NMR)
Description:	Beige powder
CAS #	Not reported
Stability of test compound:	The test material is stated to be stable for six month when stored at room temperature (date of analysis 5 January 2007)

2. Vehicle and/or positive control: Untreated diet

3. Test animals

Species:	Mice
Strain:	C57BL/6J
Age at dosing:	At least six weeks old
Weight at dosing:	19.1 to 22.4 g (males), 15.4 to 18.7 g (females)
Source:	Charles River Laboratories, St. Germain-sur-l'Arbresle, France.
Acclimation period:	At least 15 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*.
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Individually in suspended, stainless steel, wire-mesh cages.

4. Environmental conditions

Temperature:	20 to 24°C
Humidity:	40 to 70%
Air changes:	10 to 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Initiated/completed

   10 April 2007 to 31 July 2007

2. Animal assignment and treatment

   The basis for the dosing regimen of 0, 300, 600, or 1200 ppm was not specified.

   Four groups of 5 male and 5 female rats/dose were administered BYI 02960 at 0, 300, 600 or 1200 ppm in feed daily for at least 28 days, and mean doses were determined to be 0, 40, 78, and 166 mg/kg bw/day in males and 0, 47, 98 and 192 mg/kg bw/day respectively (Table 1).

   Animals were assigned to dose groups by computerised, stratified randomisation that selected animals for the study from the middle of the weight range of the available animals, such that bodyweights were within 20% of the mean bodyweight on the day of randomization. Animal housing and husbandry were in accordance with the provisions of the *Guide to the Care and Use of Laboratory Animals* (USPHS-NIH Publication No. 86-23) and "Le Guide du Journal Officiel des Communautes Europeennes L358, 18 Decembre 1986, n° 86/609/CEE du 24 Novembre 1986".

Table 1. Study design

Group	No./sex/dose	Nominal dietary dose (ppm)	Assumed mean dose level (mg/kg bw/d)*	
			M	F
1	5	0	0	0
2	5	300	(50) 40*	(59) 47*
3	5	600	(98) 78*	(122) 98*
4	5	1200	(207) 166*	(240) 192*

* 20% degradation of the test submaterial is assumed for the dose levels used in this study (the unadjusted measured mean dietary intakes are shown in brackets).
Data excerpted from page 19 of the study report.

3. Diet preparation and analysis

   The required amount of the test material was incorporated into the diet by dry mixing to achieve the nominal dietary concentrations at each dosing level. There was one preparation for each concentration. When not in use, prepared diet formulations were stored at approximately -20°C.

   The stability of BYI 02960 in the diet was not evaluated in this study. However, in a concurrent analytical study (Amir Tahmasseb and Viglieca, 2007; Study No. SA 07131, not submitted in this data package) it was reported that freezing and thawing of the diet led to problems in recovery of the test material at low dose levels (50 and 500 ppm). While in the study 28-dietary rat study (Blanck, 2012; Study No. SA 07047) recovery rates at 500 ppm were between 80% and 87% (18 days frozen and 10 days at room temperature, and 34 days frozen and 4 days at room temperature respectively) and above 90% at 5000 ppm analysed under the same conditions. Consequently, because dose levels used in this study are close to the low dose levels in the analytical study, a similar level

of stability is assumed. Therefore, determined dietary intake doses were adjusted for an assumed 20% degradation.

No analyses of homogeneity were performed on the dietary formulations.

4. Statistics

Statistical analyses of body weight, body weight gain, average food consumption, clinical chemistry and absolute and relative organ weight were carried out separately for males and females using the non-parametric Kruskal-Wallis test followed by Dunn test (2-sided) if significant ($p<0.05$) or using parametric ANOVA followed by Dunnett test (2-sided) if significant ($p<0.05$), as determined to be appropriate by Bartlett's test for homogeneity (non-parametric analyses if significant $p<0.05$ and parametric analysis if not significant).

Additionally, for body weight and average food consumption/day if Bartlett's test for homogeneity was significant ($p<0.05$) additional parametric analyses (ANOVA followed by Dunnett test (2-sided) if significant ($p<0.05$)) was performed on log transformed data in addition to non-parametric analyses of the raw mean data.

C. METHODS

1. Observations

Animals were observed at least twice daily for mortality and morbidity on weekdays and once daily on weekends. Detailed clinical observations were undertaken weekly and the nature, onset, severity, reversibility, and duration of clinical signs were recorded.

Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose faeces.

2. Body weights

All animals were weighed at study initiation and at necropsy, and on a weekly basis over the study period. Body weight change was calculated for each week and for the total study duration.

3. Food consumption, food efficiency, and daily intake

Food consumption was recorded weekly for each animal. Daily intake was calculated from food consumption and body weight data.

Food use efficiency estimates were not undertaken.

4. Clinical pathology (clinical chemistry)

Haematological and coagulation analysis was not undertaken.

Blood samples were collected following an overnight fast for all animals on study day 30 by puncture of the retro-orbital venous plexus after being anesthetized by inhalation of Isoflurane. The following clinical chemistry parameters were evaluated on the samples.

<u>Clinical chemistry parameters</u>

aspartate aminotransferase	total protein
alanine aminotransferase	albumin
alkaline phosphatase	total bilirubin
urea	creatinine
total cholesterol	triglycerides
glucose	

5. Urinalyses

Urinalyses were not undertaken.

6. Sacrifice and pathology

At termination (study day 30), animals were sacrificed by exsanguination under deep anesthesia (inhalation of isoflurane). Gross examination was performed on all animals. Organs that were weighed are listed in Table 2. Organ weight/final body weight ratios were calculated. Tissues collected from all animals and all gross lesions were processed to slides, however, while all tissues (and gross lesions) listed in Table 2 were evaluated microscopically for animals administered 0 and 1200 ppm, only the liver, kidney, and thyroid glands (and gross lesions) were evaluated microscopically at 300 and 600 ppm (Table 2).

Table 2. Organs/tissues collected for pathological examination

Organ	Organs weighed	Collected and preserved	Microscopic/histopathologic evaluation conducted
Brain	X		
Spleen	X	X	X
Liver	X	X	X
Kidneys	X	X	X
Adrenal gland	X	X	X
Thyroid gland		X	X
Parathyroid glands		X	
Testes	X	X	X
Epididymides	X	X	X
Ovaries	X	X	X
Uterus (including cervix)	X	X	X
Vagina		X	X
Gross observations		X	X

Data excerpted from pages 14 and 15 of the study report.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

No clinical signs of systemic toxicity were observed

2. Mortality

There were no unscheduled mortalities.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 1200 ppm in males compared to controls, slight decreases of 3% to 6% (not statistically significant) were seen in body weight over the study period. A loss of body weight (-0.03 g compared to 0.14 g in controls) was seen between Study Days 1 and 8, and while body weight gain was comparable to controls thereafter, a treatment related overall mean cumulative body gain decrease of 15% (not statistically significant) was observed. No treatment related effect on body weight or body weight gain was seen in females (Table 3).

Table 3. Mean body weight and body weight gain

Study day	Body weight (g)							
	Males				Females			
	Dose (ppm)							
	0	300	600	1200	0	300	600	1200
1	21±1	21±1.1 (0%)	20.8±0.9 (-1%)	20.8±1.1 (-1%)	17±0.5	16.9±0.8 (-1%)	17.2±1 (1%)	16.9±1.1 (-1%)
8	21.9±0.9	21.9±1 (0%)	21.7±1.1 (-1%)	20.6±1.1 (-6%)	18.2±0.4	18±1.1 (-1%)	17.9±0.9 (-2%)	18±0.9 (-1%)
15	22.9±0.8	22.7±0.9 (-1%)	22.7±1.1 (-1%)	21.9±1.1 (-4%)	19.2±0.9	19.4±1.4 (1%)	19.1±0.7 (-1%)	19.1±0.7 (-1%)
22	23.6±0.6	23.2±0.8 (-2%)	23.5±1.1 (0%)	22.7±0.8 (-4%)	19.8±0.6	20±1.1 (1%)	19.8±0.9 (0%)	20.1±0.9 (2%)
29	24.3±0.5	24.1±0.9 (-1%)	24.5±1.3 (1%)	23.6±0.9 (-3%)	20.8±0.4	20.8±0.8 (0%)	20.5±0.9 (-1%)	20.9±1 (0%)
Study days	Cumulative body weight gain (g)							
8	0.9±0.4	1.0±0.6 (11%)	0.8±0.5 (-11%)	**-0.2±0.7 (-122%)***	1.2±0.7	1.1±0.6 (-8%)	0.6±0.7 (-50%)	1.1±0.6 (-8%)
15	1.9±0.5	1.7±0.5 (-11%)	1.9±0.5 (0%)	1.1±1.1 (-42%)	2.2±1.1	2.5±0.9 (14%)	1.9±0.6 (-14%)	2.2±1 (0%)
22	2.6±0.6	2.2±0.6 (-15%)	2.7±0.5 (4%)	1.9±0.5 (-27%)	2.8±0.7	3.1±0.7 (11%)	2.5±0.4 (-11%)	3.2±0.9 (14%)
29	3.3±0.6	3.2±0.7 (-3%)	3.7±0.7 (12%)	2.8±0.7 (-15%)	3.8±0.7	3.9±0.5 (3%)	3.3±0.9 (-13%)	4±0.7 (5%)

* denotes value that is statistically significant at the $p \leq 0.05$ level. Values in parenthese represent the percent change from the control mean. Data excerpted from pages 33, 34, 39 and 40 of the study report.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

No treatment related effect on food consumption was seen in either sex.

Food use efficiency was not calculated by the study authors.

D. CLINICAL PATHOLOGY

1. Clinical chemistry

At 1200 ppm compared to controls, a slight though statistically significant increase in alanine aminotransferase (+43%), and alkaline phosphatase activity (+21%) was seen in females (Table 4). Noting the slight magnitude of the observed increases, and the absence of pathological correlates, these changes are not considered adverse. No treatment-related changes in clinical chemistry parameters were seen in males.

Table 4. Selected clinical chemistry parameters

	Males				**Females**			
	Dose (ppm)							
	0	**300**	**600**	**1200**	**0**	**300**	**600**	**1200**
ALAT (IU/L)	39±8	39±18 (0%)	30±2 (-23%)	29±4 (-26%)	35±5	40±4 (14%)	36±7 (3%)	**50±10 (43%)****
ALP (IU/L)	112±5	110±7 (-2%)	113±8 (1%)	118±5 (5%)	151±20	174±15 (15%)	137±11 (9%)	**183±17 (21%)***

* denotes values that are statistically significant at the $p \leq 0.05$ or ** $p \leq 0.01$ level.
Data excerpted from pages 47 and 48 of the study report.

E. SACRIFICE AND PATHOLOGY

1. Organ weight

Compared to controls, a decrease in absolute epididymis weight was seen at 300 ppm and above that did not demonstrate a clear dose relationship though was statistically significant in males at all dose levels (20-30%). Similarly in males, increases were seen in relative (to body weight and brain weight) epididymis weight at 300 ppm and above that did not demonstrate a clear dose relationship, and was statistically significant at 300 ppm (18% and 16% respectively) and 1200 ppm (23% and 26% respectively) but not at 600 ppm. These findings, which were seen in the absence of associated histopathological changes, are considered likely incidental and not treatment related.

Similarly compared to controls, a statistically significant increase in mean absolute and relative (to body weight and brain weight) spleen weight (13%, 22% and 25% respectively) in males at 300 ppm only in the absence of associated histopthological changes are considered likely incidental and not treatment related (Table 5).

No treatment related effects on organ weights were seen in females.

Table 5. Selected organ weight changes in males

Organ	Dose (ppm)			
	0	**300**	**600**	**1200**
Terminal BW (g)	20.4±0.6	20.4±0.8	20.6±1.1	19.8±0.7
Epididymides Abs (g)	0.1±0.01	**0.08±0.01 (-20%)***	**0.08±0.01 (-20%)***	**0.07±0 (-30%)****
Epididymides relBW	0.47±0.054	**0.384±0.044 (-18%)***	0.401±0.059 (-15%)	**0.364±0.029 (-23%)****
Epididymides relBrain	21.645±2.761	**18.077±2.138 (-16%)***	18.307±1.894 (-15%)	**16.084±1.198 (-26%)****
Spleen Abs (g)	0.040±0.004	**0.049±0.007 (23%)***	0.044±0.003 (10%)	0.044±0.002 (10%)
Spleen relBW	0.1956±0.0134	**0.2394±0.0285 (22%)****	0.2148±0.0082 (10%)	0.2214±0.0123 (13%)
Spleen relBrain	9.0114±0.8366	**11.2930±1.4633 (25%)****	9.8628±0.5548 (9%)	9.7787±0.4174 (9%)

*Statistically significant from control at p≤0.05 or ** p≤0.01
Data excerpted from pages 50 and 51 of the study report.

2. Gross pathology and histopathology

No treatment related effect gross or histolopathological changes were seen in either sex.

III. CONCLUSION

The study author determined the no-observed-adverse-effect-level (NOAEL) in male and female mice to be the top dose tested of 1200 ppm, given that the observed decreases in body weight gain in males were not considered adverse as they were slight and transient. Given the instability of BYI 02960 in rodent diet the actual mean NOAEL concentration was considered to be approximately 166 mg/kg bw/day for males and 192 mg/kg bw/day for females.

In males at 1200 ppm (166 mg/kg bw/day), decreased body weight gain (body weight loss of -0.03 g compared to a body weight gain of 0.14 g in controls) was seen between Study Days 1 and 8, and while body weight gain was comparable to controls thereafter, a treatment related overall mean cumulative body gain decrease of 15% (not statistically significant) was observed. This magnitude of the overall decrease in body weight gain was considered adverse by the primary reviewer. However, the EPA secondary reviewer considered the changes in the body weight gains in males not to be adverse because the absolute body weight changes were minimal (↓1% to ↓6%) and not adverse

A slight though statistically significant increase in alanine aminotransferase (+43%), and alkaline phosphatase activity (+21%) was seen in females from the 1200 ppm dose group. Noting the slight magnitude of the observed increases, and the absence of pathological correlates, these changes are not considered adverse.

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The primary evaluator agrees with the identified NOAEL in females but not in males, and considers the decreases in body weight gain between Study Days 1 and 8 and (although not statistically significant) the magnitude of the overall mean cumulative body gain decrease of 15% in males at 1200 ppm to be treatment related and toxicologically significant.

The primary evaluator determined the NOAEL for males to be 600 ppm (considered to be approximately 78 mg/kg bw/day due to instability) based on decreased body weight gain at 1200 ppm (166 mg/kg bw/day; lowest-observed-adverse-effect-level [LOAEL]). The NOAEL in females was determined to be 1200 ppm (considered to be approximately 192 mg/kg bw/day) based on the absence of adverse treatment- related effects. A LOAEL was not identified in females (being greater than 192 mg/kg bw/day). **The EPA secondary reviewer does not agree with the primary evaluator in assessing NOAEL and LOAEL. Since there was no adverse body weight effect and no other adverse effects were seen at any dose levels tested, the LOAEL could be not be established. The NOEAL was 1200 ppm (192 mg/kg bl/day) (highest dose tested).**

This study is considered useful for dose selection and provides information for evaluating the toxicity of this chemical. It is classified as acceptable/non-guideline.

Primary review: Australia
Secondary review: PMRA & US EPA

Report: Odin-Feurtet M (2008) BYI 02960. Preliminary 28-day toxicity study in the dog by dietary administration. Bayer CropScience, 355, rue Dostoievski, BP 153, 06903 Sophia Antipolis Cedex, France. Laboratory Report No.: SA 07290. 09 December, 2008. Unpublished. Study Sponsor: Bayer CropScience, Alfred Nobel Str. 50, 40789 Monheim, Germany. Sponsor identification number: Lynx-PSI N°TXRVP013. Bayer CropScience File No. M-312461-01-3. MRID 48844152. PMRA 2239321.

Guidelines: As a preliminary study, it was not designed to meet regulatory requirements.

GLP: No. This study was not conduced in compliance with GLP in that it was not subjected to specific Quality Assurance inspections and dosing suspensions were not analysed for homogeneity, stability and concentration. However, signed and dated statements stated that the study was performed according to standard operating procedures which were previously accepted and periodically inspected by the Quality Assurance unit. A signed Data Confidentiality statement was presented in the report.

Executive summary

In a 28-day dietary study (MRID 48844152), BYI 02960 was administered daily in the diet to male and female beagle dogs (2 animals/sex/concentration) at concentrations of 0, 500, 2000, or 4000 ppm (males: 0, 16.1, 61.8, and 118.3 mg/kg bw/day; females: 0, 17.8, 76.8, and 130.5 mg/kg bw/day). Parameters evaluated included body weight, body weight gain, food consumption, clinical signs, clinical pathology, ophthalmology, organ weights, and gross and microscopic pathology.

Under the conditions of this study, BYI 02960 did not produce treatment-related effects on survival, clinical signs, ophthalmoscopic examinations, clinical chemistry or urinalysis parameters. However, it should be emphasized that this study employed only 2 dogs/sex/dose; the small number of the test animal introduced great challenges in interpreting the results of this study. The usefulness of the data is rather limited.

There was a treatment related effect on the thyroid of one female dog which presented both thyroid weight increase and bilateral enlargement of the thyroid gland

At 4000 ppm, one male experienced a bodyweight loss of 0.2 kg over the study period, the other male and one female at this dose did not gain bodyweight, and lower food consumption was observed in both male and female animals. At 2000 ppm body weight loss was observed in one male over the first two weeks, and overall the body weight gain in this animal was 8.5 g compared to 18.5 and 39.8 g in control animals and was associated with lower food consumption.

At 4000 ppm, increased platelet counts were observed in both females (43 and 36%) and one male (30%), and in one female at 200 ppm (28%), relative to their pre-study value. No other treatment related changes were seen in haematology parameters. In

isolation, these treatment-related changes were not considered to be adverse.

At 4000 ppm thyroid weight data were only available for a single female, and showed an increase in absolute (0.915 g compared to 0.508 and 0.485 g in control)s and relative (0.015% compared to 0.006 and 0.008% in controls) thyroid weight. At this dose level, enlarged thyroids were observed in both females, and diffuse follicular dilation of the thyroid gland was seen in one female. The singular finding at 2000 ppm of diffuse follicular dilation of the thyroid gland in one female was not considered to be toxicologically significant. Similarly, the finding of decreased hepatic centrilobular glycogen accumulation (incidence and/or severity) at 2000 and 4000 ppm in males, and at 4000 ppm in females, was not considered adverse.

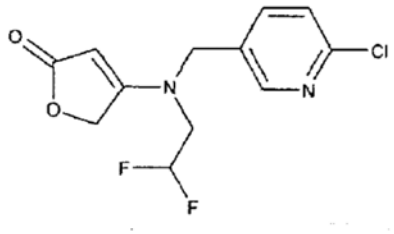
The NOAEL was 2000 ppm (62 mg/kg bw/day); LOAEL was 4000 ppm (118 mg/kg bw/day) based on d , increased absolute and relative thyroid weight and enlarged thyroid, diffuse follicular dilatation of the thyroid.

This study is considered useful for dose selection and provides certain information for evaluating the toxicity of this chemical. However, the data are limited because only 2 dogs/sex/dose were tested. It is classified as acceptable/non-guideline.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960



Lot/Batch #:	NLL 7780-44-6:
Purity:	99.5%
Description:	Dark pink powder
CAS #	Not reported
Stability of test compound:	The test material was stored at 25±5ºC in an air-tight, light-resistant container. Stated to be stable for one year if stored at room temperature (date of analysis 4 July 2008).

2. Vehicle and/or positive control: Untreated diet

3. Test animals

Species:	Dogs
Strain:	Beagle
Age at dosing:	At least eight months old
Weight at dosing:	6.7 to 9.0 kg (males), 5.2 to 7.0 kg (females)
Source:	Marshall BioResources, North Rose, New-York, USA
Acclimation period:	At least 21 days
Diet:	Certified canine meal 125C3-P1 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), Approx. 330 g/d/animal and moistened with 470 mL water prior to its provision each day.
Water:	Filtered and softened tap water, *ad libitum*
Housing:	Individually in stainless steel kennels with a floor surface area of 1.2 m^2, and where possible pair housed overnight by temporary opening of partitions. Daily supervised exercise was permitted.

4. Environmental conditions

Temperature:	18 to 21°C
Humidity:	40 to 70%
Air changes:	Approx. 15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Experimental start/completion

15 April 2008 to 05 September 2008

2. Animal assignment and treatment

The dosing regimen of 0, 500, 2000, or 4000 ppm was stated to be based on results from an oral gavage dosing study in beagle dogs where 200 mg/kg bw/d of BYI 02960 exceeded the Maximum Tolerated Dose (Kennel, 2008; Study No. SA 07291).

Four groups of 2 animals/sex/dose were administered BYI 02960 at concentrations of 0, 500, 2000, or 4000 ppm in feed daily for 28 days, and mean doses were determined to be 0, 16.1, 61.8 and 118.3 mg/kg bw/day in males and 0, 17.8, 76.8 and 130.5 mg/kg bw/day in females, respectively (Table 1).

Animals were assigned to dose groups to ensure a similar body weight distribution among groups of each sex, whilst avoiding placement of full siblings at the same dosing level. Animal housing and husbandry were in accordance with the provisions of the *Guide to the Care and Use of Laboratory Animals* (USPHS-NIH Publication No. 86-23) and and "Le Guide du Journal Officiel des Communautes Europeennes L358, 18 Decembre 1986, n° 86/609/CEE du 24 Novembre 1986".

Table 1: Study design

Dietary concentrations (ppm)	Mean achieved doses (mg/kg bw/d)[A]	
	M	F
0	0	0
500	16.1	18.8
2000	61.8	76.8
4000	118.3	130.5

[A]- Group means for each sex were calculated on weeks, 1, 2, 3 and 4, and was calculated from dietary test concentrations, food consumption and body weight data.

Data excerpted from page 22 of the study report.

3. Diet preparation and analysis

The test material was ground to a fine powder and the required amount incorporated into the diet by dry mixing to achieve the nominal dietary concentrations at each dosing level. There was one preparation for each concentration, except for the 500 ppm batch, which was re-made after achieved concentrations were found to be unsatisfactory. When not in use, prepared diet formulations were stored at approximately -18°C in light resistant containers.

All batches were subjected to analyses of achieved test material concentrations, and 500 and 4000 ppm batches were subjected to homogeneity analysis, as well as stability analysis after 28 days storage at -18ºC and 10 days at room temperature, or after a minimum of 38 days storage at room

temperature.

Homogeneity analysis found ranges of 92 to 96% of nominal concentrations, and concentration analysis found ranges of 88-95% of nominal concentrations, which the laboratory regarded as acceptable.

The test material was found the be stable in the 4000 ppm diet after at least 48 days of storage at room temperature (93% of nominal) and after 38 days storage followed by 10 days at room temperature (93% of nominal). The test material was found to be stable in the 500 ppm test diet after at least 38 days of storage at room temperature (92% of nominal) and after 28 days of frozen storage followed by 10 days at room temperature (95% of nominal).

4. Statistics

No statistical analyses were conducted since the number of test animal was only 2/sex/group.

C. METHODS

1. Observations

Animals were observed at least twice daily for mortality and morbidity on weekdays and once daily on weekends. Detailed clinical observations were undertaken daily. A detailed physical examination was undertaken once at the end of the treatment period, and included but were not necessarily limited to examination of fur and skin, eyes, ears, teeth, gum, mucous membranes, rectal temperature, gait, stance, general behaviour, chest including heart and respiratory rate, abdomen including palpation, external genitalia and mammary glands.

Daily examinations of kennels were also carried out for vomitus, blood or diarrhoea.

2. Body weights

All animals were weighed at study initiation and at necropsy, and on a weekly basis over the study period. Body weight change was calculated for each week and for the total study duration

3. Food consumption, food efficiency, and daily intake

Food consumption for each dog was measured and recorded daily during the study. Mean food consumption and test material consumption were calculated for each week and for the total study duration. Food use efficiency estimates were not provided.

4. Ophthalmology

The eyes of all animals were examined by focal illumination and indirect ophthalmoscopy prior to study start. The eyes of all surviving animals were examined again prior to scheduled sacrifice.

5. Clinical pathology (haematology, coagulation, and clinical chemistry)

Blood samples were collected from all animals at pretest and on study day 27 by puncture of the jugular vein. The following hematology, clinical chemistry, and coagulation parameters were evaluated on the samples.

Haematology and coagulation

Blood smear examination was undertaken only if abnormal haemotology results were obtained.

Red blood cell count	Haemoglobin
Reticulocyte count	Haematocrit
Platelet count	Mean corpuscular (cell) volume
White blood cell count	Prothrombin time
Differential white blood cell count	Activated partial thromboplastin time
Mean corpuscular (cell) hemoglobin	
Mean corpuscular (cell) hemoglobin concentration	

Clinical chemistry parameters

aspartate aminotransferase	total protein (in serum)
alanine aminotransferase	albumin (in serum)
alkaline phosphatase	total bilirubin
urea nitrogen	creatinine
total cholesterol	triglycerides
glucose	chloride
sodium	potassium
calcium	inorganic phosphorous
gamma-glutamyltransferase	Globulins and A/G ratio

6. Urinalyses

Urine sample were collected from all animals overnight at pretest and on study day 28. The following parameters were examined.

Quality	ketone
pH	glucose
blood	urobilinogen
bilirubin	protein
microscopic urine sediment examination	
specific gravity (refractive index)	

7. Sacrifice and pathology

At termination (study day 29 and 30), animals were tranquilized by intramuscular injection of acepromazine and sacrificed by exsanguination under deep anesthesia (pentobarbital by i.v. injection). Gross examination was performed on all animals. Organs that were weighed are listed in Table 2. Organ weight/final body weight ratios were calculated. Tissues collected from all animals and all gross lesions were processed to slides and evaluated

microscopically (Table 2).

Table 2. Organs/tissues collected for pathological examination

Tissue	Organs weighed	Collected and preserved	Microscopic/ histopathologic evaluation conducted
Adrenal gland	X	X	X
Articular surface (coxo-femoral)		X	X
Aorta		X	X
Bone with bone marrow, sternum		X	X
Bone marrow smear[(A)]		X	
Brain (cerebrum, midbrain, cerebellum, medulla/pons)	X	X	X
Epididymis	X	X	X
Esophagus		X	X
Eye (with optic nerve)		X	X
Gallbladder		X	X
Heart	X	X	X
Kidney	X	X	X
Intestines (duodenum, jejunum, ileum, cecum, colon, rectum)		X	X
Larynx/pharynx		X	
Liver	X	X	X
Lung		X	X
Lymph nodes (retropharyngeal, mesenteric)		X	X
Mammary gland		X	X
Ovary	X	X	X
Oviduct		X	X
Pancreas		X	X
Pituitary	X	X	X
Prostate	X	X	X
Salivary gland, submandibular		X	X
Sciatic nerve		X	X
Skeletal muscle		X	X
Skin		X	X
Spinal cord, (cervical, lumbar, thoracic)		X	X
Spleen	X	X	X
Stomach		X	X
Testis	X	X	X
Thymus	X	X	X
Thyroid gland (with parathyroid)	X	X	X
Tongue		X	X
Trachea		X	X
Urinary bladder		X	X
Uterus with cervix	X	X	X
Vagina		X	X
Gross lesions		X	X

A – a bone marrow smear of one rib was prepared for each animal, but were not examined for any group due to the absence of abnormal haematology findings.

Data excerpted from page 17 of the study report.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

No clinical signs of systemic toxicity were observed.

2. Mortality

There were no unscheduled mortalities.

B. BODY WEIGHT AND BODY WEIGHT GAIN

Over the study period, control animals experienced bodyweight gains of 0.5 to 1.1 kg, whereas at 4000 ppm, one male (M1159) experienced a body weight loss of 0.2 kg. The other male (M1158) and one female (F1161) at this dose level essentially did not gain any body weight (changes of -.0.05 to +0.06 kg), while the remaining female (M1160) gained 0.7 kg. At 2000 ppm body weight loss was observed in one male (M1154) over the first two weeks, and the overall body weight gain in this animal was 0.22 kg compared to 0.5 to 1.1 kg in control animals. At 2000 ppm in females and 500 ppm in both sexes, body weight gains were unaffected by treatment (Table 3).

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

At 4000 ppm over the study period, decreased food consumption was observed in all males and females compared to pre-test values and controls. At 2000 ppm, decreased food consumption was seen in both males compared to pre-test values, and the observed decreases were generally lower when compared to controls. These observations were associated with decreases in body weight gain. At 2000 ppm in females and 500 ppm in both sexes, food consumption were unaffected by treatment (Table 3).

Food use efficiency was not determined.

Table 3. Bodyweight gain and food consumption

	Males – Animal No.								Females – Animal No.							
Animal No	**1146**	**1147**	**1150**	**1151**	**1154**	**1155**	**1158**	**1159**	**1148**	**1149**	**1152**	**1153**	**1156**	**1157**	**1160**	**1161**
dose (ppm)	**0**		**500**		**2000**		**4000**		**0**		**500**		**2000**		**4000**	
Body weight gain																
BWG Day 1 to 8 (g/day)	11.43	74.29	51.43	88.57	**-2.86**	**-8.57**	**-8.57**	**-50**	25.7	41.4	32.9	44.3	34.3	42.9	21.4	0.0
BWG Day 9 to 15 (g/day)	**-1.43**	27.14	1.43	4.29	**-8.57**	10.00	**0**	11.43	25.7	37.1	44.3	15.7	8.6	5.71	15.7	**-1.4**
BWG Day 16 to 22 (g/day)	45.71	32.86	42.86	54.29	27.14	41.43	22.86	28.57	21.43	34.29	37.14	48.57	50	35.71	22.86	10.0
BWG Day 23 to 28 (g/day)	18.33	25.00	**-8.33**	26.67	18.33	10.00	**-6.67**	**-25**	**-8.33**	**0**	26.67	5.00	15	20.0	53.33	**-18.33**
Cumulative BWG Day 1 to 8 (kg)	*0.08*	*0.52*	*0.36*	*0.62*	***-0.02***	***-0.06***	***-0.06***	***-0.35***	*0.18*	*0.29*	*0.23*	*0.31*	*0.24*	*0.30*	*0.15*	***0.00***
Cumulative BWG Day 1 to 15 (kg)	*0.07*	*0.71*	*0.37*	*0.65*	***-0.08***	***0.01***	***-0.06***	***-0.27***	*0.36*	*0.55*	*0.54*	*0.42*	*0.30*	*0.34*	*0.26*	***-0.01***
Cumulative BWG Day 1 to 22 (kg)	*0.39*	*0.94*	*0.67*	*1.03*	*0.11*	*0.30*	***0.10***	***-0.07***	*0.51*	*0.79*	*0.80*	*0.76*	*0.65*	*0.59*	*0.42*	***0.06***
Cumulative BWG Day 1 to 28 (kg)	*0.50*	*1.09*	*0.62*	*1.19*	***0.22***	*0.36*	***0.06***	***-0.22***	*0.46*	*0.79*	*0.96*	*0.79*	*0.74*	*0.71*	*0.74*	***-0.05***
Food consumption (mean percent daily ration eaten)																
Pre-study	73	87	67	67	69	100	70	91	80	73	50	75	73	73	80	68
Week l	67	100	70	73	63	73	52	67	85	83	57	71	73	72	64	53
Week 2	63	100	75	67	58	86	60	76	93	88	64	75	72	73	70	56
Week 3	78	100	84	85	79	98	67	83	88	83	65	81	89	88	74	67
Week 4	69	92	76	81	73	100	72	80	85	77	72	80	83	80	75	60
Wk 1 to 4 (% change from pre-study)	***-5.1***	*12.6*	*13.8*	*14.2*	***-1.1***	***-10.8***	***-10.4***	***-15.9***	*9.7*	*13.4*	*29.0*	*2.3*	*8.6*	*7.2*	***-11.6***	***-13.2***

Body weight gain data excerpted from pages 48 and 50 of the study report () and food consumption data excerpted from page 22 of the study report (wk 1-4 % change from pre-study calculated by the evaluator).

D. CLINICAL PATHOLOGY

1. Hematology and coagulation

At 4000 ppm, increased platelet counts were observed in both females (43 and 36%) and one male (30%), relative to their pre-study values. At 2000 ppm, an increase in platelet count was noted in one female (28%, relative to its pre-study value). However, similar increases over the study period were noted in one male at 500 ppm (25%) and in both control females (15-28%). No treatment-related changes in platelet counts were observed in the 90-day (Study No. 09-S76-QQ) or 1-year (Study No. 09-C76-RZ) dietary studies in dogs with flupyradifurone. With only 2 dogs/group, interpretation of these findings is challenging. However, in isolation, these potentially treatment-related changes are not considered to be adverse. No other treatment related changes were observed in hematology parameters.

Table 4. Selected haematology parameters

males								**females**							
dose (ppm)															
0		**500**		**2000**		**4000**		**0**		**500**		**2000**		**4000**	
Platelets (10^9/L) (pre-study)															
270	293	299	234	284	443	341	468	362	251	296	334	299	390	337	411
Platelets (10^9/L) (day 27)															
271	309	290	292	300	457	369	608	418	322	347	329	319	500	482	559
per cent change in platelets pre-study d13 to d27															
0	5	-3	25	6	3	8	**30**	15	28	17	-1	7	**28**	**43**	**36**

Data excerpted from pages 59, 62, 66 and 69 of the study report.

2. Clinical chemistry

At 4000 ppm, an increase of 31% was observed in serum creatinine concentration in one female (31%) relative to its own pre-study value. As no relevant histopathological findings were seen in the kidneys, this finding in one of two females only, which was absent in males, was not considered to be treatment related.

Table 5. Selected clinical chemistry parameters

males								**females**							
dose (ppm)															
0		**500**		**2000**		**4000**		**0**		**500**		**2000**		**4000**	
Creatinine (umol/L) (day 13 pre-study)															
71	76	78	69	67	64	73	82	62	67	64	69	70	75	67	74
Creatinine (umol/L) (day 27)															
73	73	75	71	80	68	82	92	66	68	61	71	75	88	88	80
percent change in creatinine pre-study d13 to d27															

3	**-4**	**-4**	3	19	6	12	12	6	1	**-5**	3	7	17	31	8

Data excerpted from pages 74, 77, 81 and 84 of the study report.

3. Urinalysis

Urinalyses parameters were unaffected by treatment.

E. OPHTHALMOLOGY

Ophthalmological parameters were unaffected by treatment.

F. SACRIFICE AND PATHOLOGY

1. Organ weight

A sole female (F1161) at 4000 ppm had an absolute thyroid weight of 0.915 g compared to 0.508 and 0.485 g in controls. The relative (to bodyweight) thyroid weight ratio for this animal was 0.015% compared to 0.006 and 0.008% in controls. The thyroid sample for the second female at this dose (F1160) was listed as being missing. Absolute and relative thyroid weights in males were unaffected.

There were no other differences in absolute and relative organ weights between dosing and control groups.

2. Gross pathology and histopathology

Enlarged thyroid glands (observed bilaterally in animal No. F1161, and on the available singular gland in animal F1160) was noted in both females at 4000 ppm, but not in any other animal. Additionally, diffuse follicular dilation of the thyroid gland was seen in one female at 2000 and 4000 ppm. Noting the absence of a dose response, the singular finding at 2000 ppm is considered unlikely to be toxicologically significant. However, the finding at 4000 ppm seen in the presence of enlarged/increased thyroid weight cannot be so readily dismissed and is tentatively considered toxicologically significant.

Hepatic centrilobular glycogen accumulation was decreased in incidence and/or severity at 2000 and 4000 ppm in males, and at 4000 ppm in females, and while considered to be treatment related was not adverse (Table 6).

All other gross necropsy and histological findings were unrelated to the test material.

Table 6. Selected histopathology findings

N=2	Males				Females			
	Dose (ppm)							
	0	**500**	**2000**	**4000**	**0**	**500**	**2000**	**4000**
Diffuse centrilobular hepatocellular glycogen accumulation								
Minimal	-	-	1	-	2	2	2	-
Slight	2	2	-	-	-	-	-	-
Total	2 (100%)	2 (100%)	1 (50%)	-	2 (100%)	2 (100%)	2 (100%)	-
Thyroid gland, diffuse follicular dilatation (minimal):								
Total	-	-	-	-	-	-	1 (50%)	1 (50%)

Data excerpted from pages 24, 162, 164, 167, 170 of the study report.

III. CONCLUSION

The study author considered the no-observed-adverse-effect-level (NOAEL) in both sexes to be 2000 ppm (62/77 mg/kg bw/d in M/F), based on findings at 4000 ppm (118/130 mg/kg bw/d in M/F) consisting of an overall 0.2 kg body weight loss in one male over the 28 day study period, and absence of bodyweight gain in the other male and one female.

The study author did not consider the gross pathology findings of enlarged thyroid in 4000 ppm females to be treatment related, stating that thyroid weight was unaffected and no histopathological changes were seen in the thyroid.

The evaluator agrees with the identified NOAEL in females but not in males, and considers that the enlarged thyroid glands in females at 4000 ppm associated with increased absolute and relative liver weight and diffuse follicular dilatation of the thyroid are treatment related and toxicologically significant findings albeit in one sex.

The US EPA secondary reviewer considered the data to be of limited value because only 2 dogs/sex/dose were tested in this study, and the effects seemed to be confined in either one of the dogs. However, the thyroid effects seen at 4000 ppm were present in two females, and the thyroid effect in one female was bilateral. Therefore, the The NOAEL was 2000 ppm (62 mg/kg bw/day); LOAEL was 4000 ppm (118 mg/kg bw/day) based on dincreased absolute and relative thyroid weight, enlarged thyroid, and diffuse follicular dilatation of the thyroid.

This study is considered useful for dose selection and provides certain information for evaluating the toxicity of this chemical. It is classified as acceptable/non-guideline.

Primary review: Australia
Secondary review: Us EPA & PMRA

Report:	Kennel P. (2011) BCS-AA56716 (Difluoroacetic acid), Preliminary 14-day toxicity study in the rat by dietary administration. Bayer S.A.S.Bayer CropScience 355, rue Dostofevski BP 153, 06903 Sophia Antipolis Cedex, France. Report No: SA 10323. Document No: M-414152-01-2. Sponsor identification number: Lynx-PSI N°TXRVP091. September 19, 2011. MRID 48844153. Unpublished.
Guidelines:	Not applicable (only preliminary and explorative study design)
GLP	Study not performed under GLP, but laboratory GLP-certified

Executive Summary

In 14-day oral toxicity study (MRID 48844153), BCS-AA56716 (difluoroacetic acid), a metabolite of BYI 02960 (batch number BCOO 5984-4-11: a colorless liquid, 96.7% w/w purity) was administered in the diet to groups of Wistar rats (5/sex/group) for at least 14 days at concentrations of 0, 500, 2000 and 8000 ppm (0, 48, 187 and 745 mg/kg body weight/day in males; 0, 51, 201 and 800 mg/kg bw/day in females). A similarly constituted group received untreated diet and served as a control. Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight and food consumption were recorded once weekly. Hematology and clinical chemistry parameters were determined at the end of the study. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically.

BCS-AA56716 dietary administration at dose levels of 8000, 2000 and 500 ppm to males and females Wistar rats for at least 14 days induced no treatment-related changes with regard to survival, clinical signs, food consumption, hematological parameters, macroscopic observation and microscopic examination.

At 8000 ppm, mean body weight was similar to the controls in both sexes on study Day 8 and was slightly reduced by 4% in males and 3% in females on study Day 15 (not statistically significant) in comparison to the controls. This slight effect was attributable to a mean cumulative body weight gain reduced by 13% in males and 12% in females throughout the study (not statistically significant) when compared to the controls. Clinical chemistry revealed a mean glucose concentration reduced by 54% in males and 45% in females ($p < 0.01$) compared to the controls. In addition, mean urea concentration was 27% higher in females (not statistically significant) in comparison to the controls. At necropsy, mean terminal body weight was lower by 6% in males and 7% in females (not statistically significant), compared to the controls.

At 2000 ppm, mean body weight parameters were unaffected by treatment in males, whilst in females a marginal reduction in mean cumulative body weight gain was noted throughout the study (- 12% compared to the controls, not statistically significant). Clinical chemistry revealed a mean glucose concentration reduced by 41% in males and 48% in females ($p < 0.01$) compared to the controls. In addition, mean urea concentration was 25% higher in females (not statistically significant) in comparison

to the controls. At necropsy, mean terminal body weight was lower by 6% in females only (not statistically significant) compared to the controls. The change observed in males at this dose level was considered not to be adverse in view of its isolated occurrence and absence of associated histological findings.

At 500 ppm, the only changes noted for clinical chemistry consisted of a mean glucose concentration reduced by 34% in males and 42% in females ($p < 0.01$), together with mean urea concentration higher by 23% in females (not statistically significant) in comparison to the controls. These few changes were considered not to be adverse in view of their isolated occurrence and absence of associated histological findings.

Based on the results, the NOAEL was considered to be 500 ppm (51 mg/kg bw/day); LOAEL was 2000 ppm (187 mg/kg body weight/day) based decreased mean glucose concentrations (↓41% in males and 48% in females) and increased mean urea concentrations (↑25% in females) in comparison to the controls.

The results are valid, but study does not meet guideline requirements for a subchronic toxicity study.

I. Materials and Methods

A. Material

1. Test Material:	BCS-AA56716
Description:	Colorless liquid
Lot/Batch:	BCOO 5984-4-11
Purity:	96.7%%
CAS:	381-73-7
Stability of test compound:	No analysis were performed on the dietary formulations
2. Vehicle and /or positive control:	None
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age:	6 weeks approximately
Weight at dosing:	189 to 208 g for the males; 148 to 166 g for the females
Source:	R. Janvier, Le Genest St Isle, France
Acclimation period:	6 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages
Environmental conditions:	Temperature: 22 ± 2 °C
	Humidity: 55 ± 15%
	Air changes: Approximately 10 to15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study Design

1. In life dates

29 September 2010 to 20 October 2010 at Bayer CropScience, Sophia Antipolis, France.

2. Animal assignment and treatment

There were 5 rats per sex per dose group. Animals were assigned to dose groups using a randomization by weight. BCS-AA56716 was administered in the diet for 14 days to Wistar rats at the following doses: 0, 500, 2000 and 8000 ppm (equating to approximately 48, 187 and 745 mg/kg/day in males and 51, 201 and 800 mg/kg/day in females). A negative control group received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

3. Diet preparation and analysis

BCS-AA56716 was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. When not in use, the diet formulations were stored at approximately -18 °C. No analyses were performed on the dietary formulations.

Table 5.8-05: Study design

Test Group	Diet Concentration (ppm)	Animals assigned	
		Male	Female
1	0	5	5
2	500	5	5
3	2000	5	5
4	8000	5	5

4. Statistics

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

B. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). All animals were observed for clinical signs at least once daily. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight

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

3. Food consumption and compound intake

Food consumption was recorded weekly; the weekly mean achieved dosage intake for each week and for weeks 1 to 2 was calculated.

4. Clinical pathology

Blood sampling

On Study Day 16, blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane (Baxter, Maurepas, France). Blood was collected on EDTA for hematology, on clot activator (for serum) for clinical chemistry and on sodium citrate for coagulation parameters.

Hematology

Red blood cell count, haemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count were assayed using an Advia 120 (Siemens, Eragny, France).
A blood smear was prepared and stained using May-Grünwald-Giemsa method. It was examined when the results of Advia 120 determinations were abnormal.
Prothrombin time (10) was assayed on an ACL ElitePro (Instrumentation Laboratory, Paris, France).

Clinical chemistry

Any significant change in the general appearance of the serum was recorded.

Total bilirubin, glucose, urea, creatinine, total protein, albumin, total cholesterol and triglycerides concentrations, and aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed using an Advia 1650 (Siemens, Eragny, France).

5. Sacrifice and pathology

On Study Day 16, all animals from all groups were sacrificed by exsanguination whilst under deep anesthesia (Isoflurane inhalation). Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically. The following organs were weighed: Adrenal glands, brain, kidney, liver, ovary, spleen, testis, thyroid gland (with parathyroid gland), uterus (including cervix) and pituitary gland. Paired organs were weighed together. The same organs plus epididymis, vagina and macroscopic findings were sampled and fixed by immersion in neutral buffered 10% formalin with the exception of testis and epididymis that was fixed in Davidson's fixative. Histological sections were prepared for all animals in all groups and stained with hematoxylin and eosin. Histopathological examinations were performed on all tissues except for the parathyroid gland, for all animals in the control and high dose group. Kidney, liver, thyroid gland and significant macroscopic findings of all animals were examined in the intermediate dose groups.

II. Results and discussion

A. Observations

1. Clinical signs of toxicity

There were no clinical signs observed during the study.

2 .Mortality

There were no mortalities during the course of the study.

B. Body weight and body weight gain

At 8000 ppm, mean body weight was similar to the controls in both sexes on study Day 8 and was slightly reduced by 4% in males and 3% in females on study Day 15 (not statistically significant) in comparison to the controls. This slight effect was attributable to a mean cumulative body weight gain reduced by 13% in males and 12% in females throughout the study (not statistically significant) when compared to the controls.
At 2000 ppm, mean body weight parameters were unaffected by treatment in males, whilst in females a marginal reduction in mean cumulative body weight gain was noted throughout the study (- 12% compared to the controls, not statistically significant).
At 500 ppm, mean body weight parameters were unaffected by treatment in either sex.

C. Food consumption and compound intake

At 8000, 2000 and 500 ppm, mean food consumption was unaffected by treatment in either sex. The mean achieved dose levels of BCS-AA56716 (difluoroacetic acid) at 500, 2000 and 8000 ppm received by the animals during the study were 48, 187 and 745 mg/kg/day in males, respectively, and 51, 201 and 800 mg/kg/day in females, respectively.

D. Clinical pathology

1. Hematology

No treatment-related change was noted at any dose level in either sex. The few differences observed, even if statistically significant, were considered to be incidental in view of the low magnitude of the change and/or absence of dose-effect relationship.

2. Clinical chemistry

Lower mean glucose concentrations were noted at all dose levels in both sexes.
In addition at 8000, 2000 and 500 ppm, slightly higher mean urea concentrations were seen in females (+ 27%, + 25% and + 23%, respectively). Since these changes were not statistically significant and as no relevant variation was noted in creatinine concentrations or at the histological examination, they were considered not to be adverse effects.
Lower mean total bilirubin concentrations (- 45%, $p < 0.05$) were also noted at 8000 and 500 ppm. In the absence of a dose-effect relationship, these variations were considered not to be treatment related.

Table 5.8-06: Significant clinical chemistry changes [Mean ± standard deviation (% change when compared with controls)]

Dose level of BCS-AA56716 (ppm)	0	500	2000	8000
Male				
Glucose (mmol/l)	5.47 ± 0.33	3.62 ± 0.50** (- 34%)	3.25 ± 0.81** (- 41%)	2.50 ± 0.32** (- 54%)
Urea	5.43 ± 0.71	6.46 ± 0.68 (+ 19%)	5.91 ± 0.63 (+ 9%)	5.78 ± 0.71 (+ 6%)
Total bilirubin (µmol/l)	0.9 ± 0.2	0.6 ± 0.2 (- 33%)	0.5 ± 0.2 (- 44%)	0.7 ± 0.2 (- 22%)
Female				
Glucose (mmol/l)	4.89 ± 0.33	2.86 ± 0.42 (- 42%)	2.53 ± 0.56** (-48%)	2.68 ± 0.43** (- 45%)
Urea	5.31 ± 0.38	6.54 ± 0.87 (+ 23%)	6.63 ± 0.59 (+ 25%)	6.77 ± 1.37 (+ 27%)
Total bilirubin (µmol/l)	1.1 ± 0.3	0.6 ± 0.3 * (- 45%)	0.9 ± 0.2 (- 18%)	0.6 ± 02* (- 36%)

* : $p < 0.05$ ** : $p < 0.01$

E. Sacrifice and pathology

1. Organ weights

At 8000 ppm, lower mean terminal body weight was observed in males (- 6%, not statistically significant) when compared to the controls. At 8000 and 2000 ppm, lower mean terminal body weight was observed in females (- 7% and - 6%>, respectively, not statistically significant) when compared to the controls.

2. Gross and histopathology

All changes were considered to be incidental and not treatment-related.

III. Conclusions

In conclusion, the dietary level of 2000 ppm (corresponding to 187 mg/kg body weight/day) was considered to be a NOAEL in males, and the dietary level of 500 ppm (corresponding to 51 mg/kg body weight/day) was considered to be a NOAEL in females.

Primary review: Australia
Secondary Review: US EPA

IIIA 7.6.1 Dermal absorption, *in vivo* in the rat

Report: Odin-Feurtet M (2010) BYI 02960 (SL200) *In vivo* dermal absorption study in the male rat. Bayer S.A.S., Bayer CropScience, Newark, 355, rue Dostoïevski Delaware, USA. Laboratory Report No.: SA 09255. November 30, 2010. MRID 48844557. Unpublished.

Guidelines: OECD TG 427 (2004)
OECD 28 (2004),
ECC Sanco/222/2000 Rev.7 (2004)

Deviations: None

GLP: Yes. Signed statements of GLP Compliance, Data Confidentiality, and Quality Assurance were submitted in the study report.

Executive summary:

In an *in vivo* dermal absorption study with a [pyridinylmethyl-^{14}C]-BYI02960 200g/L soluble concentrate (SL) formulation, 7 to 9 week-old male Wistar Rj: WI (IOPS HAN) rats in groups of 4/concentration/timepoint (MRID 48844557) over four sacrifice timepoints of 8, 24, 72, or 168 hours received either the undiluted 200 g/L SL formulation, or 0.625 g/L or 0.1 g/L aqueous dilutions for eight hours. Absorption was followed using [^{14}C]-BYI02960, which was uniformly blended into the formulations prior to application. Animals were lightly anaesthetised by an unspecified method and two plastic 'protective saddles' were secured to define the application site for the test substance The formulated products were then applied dose to a 2 x 6 cm^2 shaved area of dorsal skin as two aliquots of 60 µL (total of 120 µL) to each animal in the 12 groups (N = 4/group), and the exposed skin area and protective saddles were covered in perforated plastic as a semi-occluded cover not touching the skin, and held in place over the saddles with surgical tape (~3 x 4 cm). After eight hours, covers, saddles,and tape were removed and wiped down by swabs and distilled water. The application sites were swabbed with 1% v/v Tween 80 in PBS until no more radioactivity was detected. If necessary, the treated skin was shaved with the shavings being retained for analysis. The skin was then tape stripped until the epidermis appeared shiny. The 8 hour sampling group animals were sacrificed, while animals retained for the subsequent timepoints received new protective saddles and covers.

Urine, faeces, and cage wash samples were collected individually at 0 to 8, 8 to 24, and 24-hour intervals thereafter up to the time of final sacrifice. At sacrifice, animals were subjected to terminal anaethesia by isoflurane, and exsanguinated *via* cardiac puncture. Application site skin, surrounding skin (within 1 cm perimeter, distant skin, and cardiac blood were collected, and along with skin swabs, tape strips, covers, saddle, and tape were retained for analysis of radioactivity by LSC using a Packard 1900 TR counter.

Non-absorbed dose was regarded to consist of radiolabel in dressings, surface tape strips (first two strips), and fur. Radioabel in urine, faeces, cage washes, non-treated skin and carcass was regarded as being directly absorbed. Radiolabel in stratum corneum, and skin, including surrounding skin were regarded as being potentially absorbable.

For the 200 g/L neat product, the mean total absorbable fractions were 21.78, 19.63, 17.00, and 19.18% of the applied dose for the 8, 24, 72, and 168 hour groups, respectively. Of these totals, the directly absorbed component was 2.27, 3.88, 4.39, and 8.14% of total applied dose, respectively. The test substance readily distributed to the surrounding skin, reaching 15.16% of total applied dose at 8 hours, and steadily falling to reach 7.69% of total applied dose by 168 hours. The fraction in the non-treated skin reached a maximum of 2.71% of applied dose at 72 hours, falling to 2.3% of applied dose at 168 hours. The proportion in the stratum corneum varied over time, being 1.05% at 8 hours, reaching a maximum of 4.14% of total applied dose at 24 hours, falling to 0.71% of applied dose at 72 hours, and increasing to 1.34% at 168 hours. The urine was the predominant route of excretion, containing a mean total of 2.18% of applied dose at 168 hours, while faeces contained a mean total of 0.94%. Mean total radiolabel recovery ranged from 101.1 to 113.4% of that applied dose for the 8, 24, 72, and 168 hour sacrifice groups respectively.

For the 0.625 g/L diluted product, the mean total absorbable fractions were 9.26, 8.28, 9.66, and 5.53% for the 8, 24, 72, and 168 hour exposure groups, respectively. Of these totals, the directly absorbed component was 2.21, 1.31, 2.18, and 2.24% of total applied dose, respectively. In contrast to the 200 g/L undiluted formulation, distribution to the surrounding skin was minimal, being only 0.307% of total applied dose at 8 hours, 0.345% at 24 hours and rising to 0.577% at 72 hours, and falling to reach 0.154% of total applied dose by 168 hours. The proportion in the non-treated skin was also far lower than that obtained with the 200 g/L formulation, reaching a maximum of 0.473% of applied dose at 72 hours, and falling to 0.198% of applied dose at 168 hours. The proportion in the stratum corneum was 4.86% at 8 hours and 5.96% at 24 hours, reaching a maximum at 72 hours of 6.41% of applied dose, and falling to 2.71% at 168 hours. The urine was the predominant route of excretion, containing a mean total of 1.10% of applied dose at 168 hours, while faeces contained a mean total of 0.3%. Mean total radiolabel recovery was 99.84, 99.36, 92.30, and 95.59% of total applied dose at the 8, 24, 72, and 168 hour sacrifice groups, respectively.

For the 0.1 g/L diluted product, the mean total absorbable fractions were 16.64, 10.32, 18.20, and 20.57% for the 8, 24, 72, and 168 hour exposure groups, respectively. Of these totals, the directly absorbed component was 4.32, 2.78, 5.28, and 8.12% of total applied dose, respectively. Similar to the 0.625 g/L formulation and in contrast to the 200 g/L formulation, distribution to the surrounding skin was low, being 1.53% of total applied dose at 8 hours, 0.410% at 24 hours, 0.400% at 72 hours, and rising slightly to 0.734% of total applied dose by 168 hours. The proportion in the non-treated skin was between the relatively low levels for the 0.625 g/L formulation and the high levels of the 200 g/L formulations, with a maximum of 1.551% at 8 hours, and falling to 0.794% of applied dose at 168 hours. The proportion in the stratum corneum was 8.74% at 8 hours and 6.26% at 24 hours, and as with the other concentrations, maximum levels were reached at 72 hours, at 11.69% of applied dose, and falling to slightly to 10.37% at 168 hours. The relative level of total applied dose in the stratum corneum at 168 hours was therefore 3.8-fold and 7.7-fold higher than those obtained with the 0.625 g/L and 200 g/L formulations, respectively. The urine was again the predominant route of excretion, containing a mean total of 3.00% of applied dose at 168 hours, while faeces contained a mean total of 0.443% for this timepoint. Mean total radiolabel recovery was

100.5, 95.8, 102.9, and 95.6% of total applied dose at the 8, 24, 72, and 168 hour sacrifice groups, respectively.

The last available layers of stratum corneum (tape strips 12 or 9) for the animals with the highest absorption values in each of the three 168 hour concentration groups all contained radiolabel, albeit at levels 32 to 64% lower than that on the first (surface) tape strips. When considered with the observed urinary excretion of radiolabel at 168 hour study termination, this finding demonstrated that dermal absorption of applied test material was potentially continuing up to and including the 168 hour timepoint.

The study authors stated that as a conservative estimate, the amount of BYI 02960 potentially absorbable following an 8-hour exposure ranged from 17.0 to 21.8% for the 200 g/L neat product, 5.54% to 9.66% for the 0.625 g/L intermediate dilution, and from 10.3% to 20.6% for the highly diluted 0.1 g/L formulation, based on group mean values.

The evaluator has considered that, due to the basis of high intragroup variability (standard deviation exceeding 25% of the mean), individuals in each group with the highest levels of dermal absorption were considered to be more representative of potential intraspecies variation compared to group means. On an individual animal basis, the highest absorptions of test substance were 29.9, 9.38, and 23.9% for the concentrate, 0.625 g/L, and 0.1 g/L dilutions, respectively.

In summary, following a 8-hour *in vivo* dermal exposure to [pyridinylmethyl-^{14}C]-BYI02960 200g/L SL formulation and dilutions, the maximal absorbable dose was 29.9, 9.38, and 23.9% for 200 g/L (neat), 0.625 g/L, and 0.1 g/L dilutions, respectively. The dermal absorption data from both *in vivo* and *in vitro* dermal absorption studies were used to estimate the *in vivo* dermal absorption factor for humans; the estimated factors were 42.1, 3.32 and 17.2% for BYI02960 200g/L SL concentrate, 0.625 g/L, and 0.1 g/L dilutions, respectively.

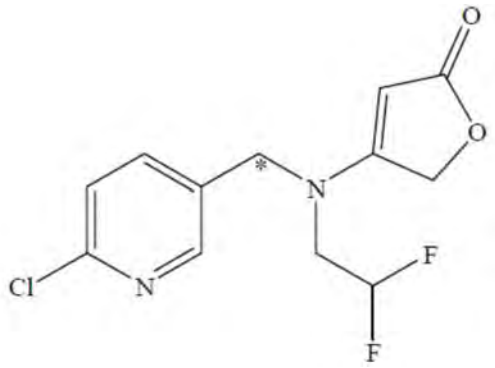
However, US EPA secondary reviewer does not agree with the conclusion concerning the human dermal absorption factor because there are no data for the exposure duration beyond 24 hours in the in-vitro human and rat skin studies. Therefore, the US EPA determines that the appropriate estimate for the potential human dermal absorption factor to be 7.42 % based on the data on the 24-hour exposure period and most dilute administered concentration (0.1 g/l).

This study is reliable (acceptable) and meets the data requirements for an *in vivo* dermal absorption study (OECD TG 427 [2004]).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: BYI 02960
   Lot/Batch #: NLL 7780-47-4
   Purity: 99.4% w/w (active constituent, Oct 2010. HPLC, UV)
   200 g a.c./L (undiluted concentrate)
   0.625 g a.c./L (intermediate field application rate)
   0.1 g a.c./L (dilute field application rate)
   Description: Liquid
   CAS #: None for the test formulation
   Not reported for the active constituent
   Stability of test compound: Stated to be stable under storage conditions (darkness at -18°C).
2. Radiolabel test material: [pyridinylmethyl-^{14}C] BYI 02960 ('BYI 02960*')



* Denotes position of the radiolabel.

   Lot/Batch #: Sample ID: KATH 6429
   Radiochemical purity: 99% (active constituent HPLC, radioactivity detector)
   98% (200 g/L dilution, HPLC RP)
   Specific activity: 4.37 MBq/mg (active constituent), 0.0185 MBq/mg (after radiodilution)
   3.54 MBq/mL (200 SL formulation), 2.76 MBq/mL (0.625 g/L dilution), 0.438 MBq/mL (0.1 g/L dilution).
   Description: Not reported
   Density: 1.138 g/mL (200 SL formulation)
   1.0 g/mL (0.625 g/L dilution)
   1.0 g/mL (0.1 g/L dilution)
   Stability of test compound: Stated to be stable under storage conditions (darkness at -18°C). The 200 g/L and 0.625 g/L dilutions were reported to have passed freeze thaw stability tests.
3. Formulation blank: BYI 02960* was radiodiluted with BYI 02960 with following co-precipitation, and mixed with the blank formulation until homogeneous to produce the 200 SL concentrate. The concentrate was further mixed with water to produce the 0.625 g/L and 0.1 g/L field application formulations (aqueous dilution). The 200 SL concentrate, and 0.625 and 0.1 g/L dilutions were stored at -18°C, thawed and homogenised for 1 to 2

		hours prior to application. Storage conditions for the blank formulation were not reported.
4.	Vehicle:	Water for the aqueous dilutions
5.	Test animals	
	Species:	Rat
	Strain:	Wistar Rj: WI (IOPS HAN)
	Sex	Males
	Age at dosing:	7 to 9 weeks old
	Weight at dosing:	260 to 375 g
	Source:	R. Janvier, Le Genest St Isle, France
	Acclimation period:	At least 14 days
	Diet:	Irradiated diet A04C-10 (from S.A.F.E. Scientific Animal Food and Engineering, Augy, France), *ad libitum*.
	Water:	Tap water, *ad libitum*
	Housing:	Individually housed in metabolism units (Jencon's Metabowls Mk III or Radleys), which comprised of a metabowl unit for separate collection of urine and faeces.
4.	Environmental conditions	
	Temperature:	22±2°C
	Humidity:	55±15%
	Air changes:	10 to 15/hour
	Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

   04 May to 26 October 2010

2. Animal assignment and treatment

   On the day prior to dermal dosing, the back and shoulders of each animal were shaved free of hair. Immediately prior to dosing, animals were lightly anaesthetised by an unspecified method and two plastic 'protective saddles' were secured using Cyanoacrylate adhesive to define the application site for the test substance (approx. total area of 2 x 6 cm^2).

   Doses applied dose and measured parameters are summarised in Table KIIIA1 7.6.1/01-1:

Table KIIIA1 7.6.1/01-1: Skin dose levels and sacrifice schedules

Group [N=4]	Concentration of BYI 02960	Skin dose level of BYI 02960 (µg/cm^2) [µg]	Time of sacrifice (Study Hour)	kBq/rat [nominal]	Animal IDs
1	200 g/L	1920 to 2080 [*2200 to 2500*]	8	412 to 463 [425]	UM1M0002 to 0005
2			24		UM2M0006 to 0009
3			72		UM3M0010 to 0013
4			168		UM4M0014 to 0017
5	0.625 g/L	6.08 to 6.42 [*73 to 77*]	8	319 to 334 [331]	UM5M0018 to 0021
6			24		UM6M0022 to 0025
7			72		UM7M0026 to 0029
8			168		UM8M0030 to 0033
9	0.1 g/L	0.975 to 0.992 [*11 to 11.9*]	8	51 to 52 [53]	UM9M0034 to 0037
10			24		UM10M0038 to 0041
11			72		UM11M0042 to 0045
12			168		UM12M0046 to 0049

Four sacrifice time intervals for all three dosing concentrations were included in this study, at 8, 24, 72, or 168 hours after initiation of dermal exposure.

3. Dose formulation and analysis

The BYI02960 200 g/L SL undiluted concentrate formulation was prepared by co-dissolution of [pyridinylmethyl-^{14}C] BYI02960 with non-radiolabeled BYI02960 following co-precipitation. The labelled and non-labelled test substances were then vortex homogenised to complete dissolution.

The 0.1 and 0.625 g/L SL undiluted concentrate formulations were prepared without radiodilution. The labelled test substances were prepared by dilution of an aliquot of samples with appropriate volumes of water, followed by vortex homogenisation and homogenised to complete dissolution.

For all three formulations, inspection for precipitates was undertaken.

The formulated dilutions were stored at <-18°C.

The homogeneity and amount of radiolabeled BYI02960 (µCi/g) in each formulated dose was determined by subjecting aliquots of the prepared dose to radiolabel analysis by liquid scintillation counting (LSC). The concentration of BYI02960 in each dose formulation was determined chromatographically by HPLC. The results of homogeneity and concentration analyses were used to calculate the specific activity of radiolabeled BYI02960 (µCi/mg) for the formulated doses. The radiochemical purity of the neat radiolabeled BYI02960 and the stability of radiolabeled BYI02960 in the prepared dose formulations were determined by HPLC-radiochromatography.

4. Dosing

On the day of dosing, the test substance at the relevant concentrations was applied dose as two aliquots of 60 µL (total of 120 µL) to each animal in the

12 groups (N = 4/group). The exposed skin area and protective saddles were covered in perforated plastic as a semi-occluded cover not touching the skin, and held in place over the saddles with surgical tape (~3 x 4 cm). The applied dose formulation remained in contact with the skin for eight hours, at which covers were removed, and they and the application sites were swabbed with natural sponge swabs soaked in newly prepared 1% v/v Tween 80 in PBS until no more radioactivity on swabs was detected by Geiger counter. Animals retained for analysis beyond eight hours (all but groups 1, 5, 9) received new covers over protective saddles to prevent radiolabel loss from desquamation.

5. In-life sample collection

At time of each sampling, cages were washed with distilled water. Urine, faeces, and cage wash samples were collected individually at 0 to 8, 8 to 24, and 24-hour intervals thereafter up to the time of sacrifice (up to study hour 168). The 8 hour (Groups 1, 5, 9), 24 hour (Groups 2, 6, 10), 72 hour (Groups 3, 7, 11), and 168 hour (Groups 4, 8, 12) skin swabs, were collected. All samples were stored in the dark at -20°C until analysis.

6. Sacrifice

At the time of sacrifice, animals were subjected to terminal anaesthesia by isoflurane, and exsanguinated *via* cardiac puncture. Cardiac blood was stored in vials containing lithium heparin and retained for analysis of radioactivity. Perforated plastic covers, protective saddles and tape were wiped down by swabs and distilled water. The treated skin was swabbed, shaved if necessary with the shavings being retained for analysis, and tape stripped by applying adhesive tape (CILS, France) for five seconds to the treated site and carefully removing it against the direction of hair growth. The stripping was repeated until the epidermis appeared shiny, indicating complete removal of the stratum corneum. The remaining skin at the site of application, surrounding skin defined as 1 cm perimeter around the dosing site, and distant skin (as a control) were collected and stored.

Cages were then washed with distilled water and 'appropriate' organic solvent (not reported). The time of termination surrounding swabs, carcass, cardiac blood, untreated skin, fur (dose site), treated skin, stratum corneum, surrounding skin, and dressings including saddle, gauze, tape, and cover, were collected and stored in the dark at -20°C if not analysed immediately.

Non-absorbed dose was regarded to consist of radiolabel in dressings, surface tape strips (first two strips), and fur. Radioabel in urine, faeces, cage washes, non-treated skin and carcass was regarded as being directly absorbed. Radiolabel in stratum corneum, and skin including surrounding skin were regarded as being potentially absorbable.

7. Sample analysis

All samples were thawed (if stored), and weighed prior to analysis. Faeces and cardiac blood were combined with a small quantity of cellulose powder and combusted (Packard Model 387 Tr-Carb), and the resulting CO_2 generated was

mixed with scintillation cocktail (Permafluor®, 12 mL) prior to radiolabel assay. Cage debris if present were analysed by a 'suitable method' depending on their nature. Faeces was homogenised in distilled water and analysed using a top drive homogeniser (Ultra-Turrax), dried, and combusted directly. Whole blood was thawed, dried, and combusted directly. Swabs including the final three swabs at eight hours and termination were solubilised using Soluene®. Tape strips were solubilised using tetrahydrofuran. Skin samples and carcass were solubilised in alcoholic potassium hydroxide. The saddle, surgical tape, cover, and fur from treated skin prior to stripping were soaked in acetonitrile, and the washings weighed and used for analysis. In the case of urine, skin swabs X-Y-Z (i.e. the last three skin swabs at 8h and respective group terminations at 24, 72 and 168 hours), tape strips, and fur, scintillation fluid was added directly to the solubilised samples and subjected to LSC analysis. For all other samples, duplicate aliquots were taken and added to scintillation fluid, except in the case of faeces, whole blood, and skin swabs, which were analysed as triplicate aliquots.

Radioactivity was measured by liquid scintillation counting for 10 minutes or for 2 sigma per cent using a Packard 1900 TR counter. Quenching effects were determined using an external standard and the spectral quench parameter method. Efficiency correlation curves were prepared for each scintillation cocktail and regularly checked using [^{14}C]-n-hexadecane standards. Scintillation counting was recalibrated when deviations greater than 2% were seen in known standards. The limit of detection was specified to be double the background values measured in blank samples.

8. Statistics: Calculations were undertaken using the Debra 5.2a software and Microsoft Excel.

II. RESULTS AND DISCUSSION

A. RADIOCHEMICAL PURITY, CONCENTRATION AND STORAGE STABILITY

The purity of radiolabeled BYI02960 was $\geq$99%. Analyses confirmed that BYI02960 was present in the dosing formulations at the appropriate concentrations and with the appropriately amount of radiolabeled test substance. [^{14}C]-BYI02960 and the formulations were shown to be stable under the conditions of this study.

B. ABSORPTION OF BYI02960 200 SL UNDILUTED CONCENTRATE

For the 200 g/L neat product, the mean total absorbable fractions were 21.78±7.12, 19.63±4.57, 17.00±3.638, and 19.18±8.628% for the 8, 24, 72, and 168 hour groups, respectively. Of these totals, the directly absorbed component was 2.270±1.82, 3.880±2.23, 4.390±2.011, and 8.140±5.39% of total applied dose, respectively. The test substance readily distributed to the surrounding skin, reaching 15.16% of total applied dose at 8 hours, and steadily falling to reach 7.69% of total applied dose by 168 hours. The fraction in the non-treated skin reached a maximum of 2.71% of applied dose, at 72 hours, falling to 2.3% of applied dose at 168 hours. The proportion in the stratum corneum varied over time, being 1.05% at 8 hours, reaching a maximum of 4.14% of total applied dose at 24 hours, falling to 0.71% of applied dose at 72 hours, and increasing to 1.34% at 168 hours. The data is consistent with potential transfer between the systemic circulation and skin

layers. Overall, the evaluator considers that there was insufficient evidence to exclude the possibility that the stratum corneum fraction or other skin compartment would not be bioavailable.

The urine was the predominant route of excretion, containing a mean total of 2.18% of applied dose at 168 hours, while faeces contained a mean total of 0.94%. The fraction recovered from the cage wash increased steadily over time, reaching 2.0% of total applied dose at 168 hours. For all groups, washings of the skin surface contained the major portion of the applied dose, being 64.0, 67.8, 60.0, and 64.4% at the 8, 24, 72, and 168 hour groups, respectively. Mean total radiolabel recovery ranged from 101.1 to 113.4% of that applied dose for the 8, 24, 72, and 168 hour sacrifice groups respectively (Table KIIIA1 7.6.1/01-2).

Table KIIIA1 7.6.1/01-2: Recovery of total radioactivity up to 168 hours following a 8-hour topical exposure to a 200 g/L undiluted concentrate [1920 to 2080 µg a.c./cm^2 skin] of BYI02960 in SYP13527 (up to 168-hours post initiation of exposure)

	Data expressed as per cent of applied dose			
	Termination hour for group [N=4/group/timepoint]			
Absorption:	**8**	**24**	**72**	**168**
Directly absorbed dose				
Urine	0.011±0.008	0.207±0.117	0.776±0.434	2.177±0.536
Faeces	ND	0.070±0.034	0.158±0.077	0.944±0.618
Cage wash	0.015±0.021	0.080±0.051	0.223±0.113	2.044±2.248
Cardiac blood	0.009±0.003	0.012±0.004	0.004±0.006	0.013±0.010
Non treated skin**[A]**	1.313±1.473	2.679±2.121	2.713±1.529	2.300±2.024
Carcass	0.922±0.372	0.831±0.531	0.515±0.264	0.662±0.315
Total directly absorbed	**2.270± 1.822**	**3.880±2.227**	**4.390±2.011**	**8.140±5.385**
Absorbable dose				
Directly absorbed dose	**2.270±1.822**	**3.880±2.227**	**4.390±2.011**	**8.140±5.385**
Stratum corneum **[B]**	1.048±0.339	4.143±1.838	0.711±0.384	1.342±0.888
Treated skin **[C]**	3.305±1.656	2.414±1.701	1.324±0.902	2.003±1.131
Surrounding skin **[D]**	15.157±8.864	9.192±4.314	10.573±3.386	7.694±4.122
Total absorbable	**21.78±7.121**	**19.63±4.570**	**17.00±3.638**	**19.18±8.628**
Unabsorbed dose				
Skin swabs (8 hr)	64.043±15.281	67.812±6.639	60.045±8.865	64.399±16.107
Terminal swabs	NS	0.326±0.225	2.172±1.090	1.064±0.816
Surrounding swabs	19.316±10.376	5.925±4.468	8.622±4.806	1.292±1.485
Tape strips 1-2	0.330±0.081	0.770±0.706	0.289±0.143	0.427±0.280
Fur	0.056±0.078	NS	1.102±1.053	0.584±0.100
Dressings	7.842±6.201	7.892±2.257	12.466±5.657	14.108±9.127
Total non-absorbed	**91.59±3.635**	**82.73±3.908**	**84.70±6.138**	**81.87±10.904**
Total recovered	113.37±7.889	102.35±4.631	101.70±5.080	101.05±2.397

ND = not detected. NS = no sample

Data excerpted from table on pages 10, 24 & 30 of the study report.

'A' – denotes sum of radioactivity in skin distant from the site of test substance application. 'B' – denotes sum of radioactivity in tape strips excluding number 1 & 2 which were considered to be non-absorbed dose. 'C'– denotes sum of radioactivity found in treated skin excluding after tape-stripping procedure. 'D'– denotes sum of radioactivity in skin adjacent to the treated skin.

C. ABSORPTION OF THE BYI02960 0.625 G/L DILUTION

For the 0.625 g/L diluted product, the mean total absorbable fractions were 9.26±4.98, 8.28±4.03, 9.66±3.08, and 5.5±2.70% for the 8, 24, 72, and 168 hour groups, respectively. Of these totals, the directly absorbed component was

2.21±0.52, 1.31±0.16, 2.18±0.31, and 2.24±0.062% of total applied dose, respectively. In contrast to the 200 g/L undiluted formulation, distribution to the surrounding skin was minimal, being only 0.307% of total applied dose at 8 hours, 0.345% at 24 hours and rising to 0.577% at 72 hours, and falling to 0.154% of total applied dose by 168 hours. The proportion in the non-treated skin was also far lower than that obtained with the 200 g/L formulation, reaching a maximum of 0.473% of applied dose at 72 hours, and falling to 0.198% of applied dose at 168 hours. The proportion in the stratum corneum was 4.86% at 8 hours and 5.96% at 24 hours, reaching a maximum at 72 hours of 6.41% of applied dose, and falling to 2.71% at 168 hours.

The urine was the predominant route of excretion, containing a mean total of 1.098% of applied dose at 168 hours, while faeces contained a mean total of 0.3%. The fraction recovered from the cage wash increased steadily over time, reaching 0.293% of total applied dose at 168 hours.

For all groups, washings of the skin surface contained the major portion of the applied dose, being 88.10, 78.18, 71.85, and 78.81% at the 8, 24, 72, and 168 hour groups, respectively. Mean total radiolabel recovery was 99.84, 99.36, 92.30, and 95.59% of total applied dose at the 8, 24, 72, and 168 hour sacrifice groups, respectively (Table KIIIA1 7.6.1/01-3).

Table KIIIA1 7.6.1/01-3: Recovery of total radioactivity up to 168 hours following a 8-hour topical exposure to a 0.625 g/L dilution [6.08 to 6.42 µg a.c./cm^2 skin] of BYI02960 in SYP13529 (up to 168-hours post initiation of exposure)

	Data expressed as per cent of applied dose			
	Termination hour for group [N=4/group/timepoint]			
Absorption:	**8**	**24**	**72**	**168**
Directly absorbed dose				
Urine	0.258±0.228	0.337±0.165	0.835±0.284	1.098±0.074
Faeces	0.028±0.033	0.088±0.051	0.240±0.096	0.300±0.085
Cage wash	0.145±0.063	0.200±0.168	0.190±0.066	0.293±0.085
Cardiac blood	0.017±0.006	0.026±0.007	0.001±0.003	0.007±0.009
Non treated skin**[A]**	0.441±0.233	0.316±0.114	0.473±0.497	0.198±0.073
Carcass	1.326±0.258	0.342±0.127	0.444±0.117	0.346±0.091
Total directly absorbed	**2.214±0.516**	**1.309±0.161**	**2.184±0.307**	**2.241±0.062**
Absorbable dose				
Directly absorbed dose	**2.214±0.516**	**1.309±0.161**	**2.184±0.307**	**2.241±0.062**
Stratum corneum **[B]**	4.860±4.441	5.957±3.887	6.414±3.306	2.706±2.455
Treated skin **[C]**	1.875±0.712	0.669±0.164	0.489±0.333	0.434±0.310
Surrounding skin **[D]**	0.307±0.084	0.345±0.055	0.577±0.348	0.154±0.061
Total absorbable	**9.256±4.982**	**8.280±4.026**	**9.663±3.083**	**5.534±2.703**
Unabsorbed dose				
Skin swabs (8 hr)	88.10±4.535	78.18±2.030	71.85±6.708	78.81±3.215
Terminal swabs	NS	10.37±7.791	4.769±1.529	6.918±4.730
Surrounding swabs	1.156±0.268	0.164±0.121	0.310±0.249	0.158±0.012
Tape strips 1-2	1.259±0.429	2.149±1.904	4.144±3.062	1.613±0.673
Fur	NS	NS	1.165±1.377	1.034±2.068
Dressings	0.065±0.062	0.223± 0.104	0.400±0.270	1.526±2.570
Total non-absorbed	**90.58±4.863**	**91.07±4.367**	**82.63±3.960**	**90.06±3.323**
Total recovered	99.84±0.453	99.36±0.457	92.30±1.33	95.59±2.595

ND = not detected. NS = no sample

Data excerpted from table on pages 26 and 31 of the study report.

'A' – denotes sum of radioactivity in skin distant from the site of test substance application. 'B' – denotes sum of radioactivity in tape strips excluding number 1 & 2 which were considered to be non-absorbed dose. 'C'– denotes sum of radioactivity found in treated skin excluding after tape-stripping procedure. 'D'– denotes sum of radioactivity in skin adjacent to the treated skin.

D. ABSORPTION OF THE BYI02960 0.1 G/L DILUTION

For the 0.1 g/L diluted product, the mean total absorbable fractions were 16.64±5.167, 10.32±3.852, 18.20±2.704, and 20.57±3.152% for the 8, 24, 72, and 168 hour exposure groups, respectively. Of these totals, the directly absorbed component was 4.323±1.676, 2.783±0.724, 5.278±0.736, and 8.120±1.019% of total applied dose, respectively. Similar to the 0.625 g/L formulation and in contrast to the 200 g/L formulation, distribution to the surrounding skin was low, being 1.533% of total applied dose at 8 hours, 0.410% at 24 hours, 0.400% at 72 hours, and rising slightly to 0.734% of total applied dose by 168 hours. The proportion in the non-treated skin was between the relatively low levels for the 0.625 g/L formulation and the high levels of the 200 g/L formulations, with a maximum of 1.551% at 8 hours, and falling to 0.794% of applied dose at 168 hours. The proportion in the stratum corneum was 8.74% at 8 hours and 6.26% at 24 hours, and as with the other concentrations, maximum levels were reached at 72 hours, at 11.69% of applied dose, falling slightly to 10.37% at 168 hours. The relative level of total applied dose in the stratum corneum at 168 hours was therefore 3.8-fold

and 7.7-fold higher than those obtained with the 0.625 g/L and 200 g/L formulations, respectively.

For the 0.1 g/L diluted product, the urine was again the predominant route of excretion, containing a mean total of 3.004% of applied dose at 168 hours, while faeces contained a mean total of 0.443% applied dose at this timepoint. The fraction recovered from the cage wash increased steadily over time, reaching 0.698% of total applied dose at 168 hours.

For all groups, washings of the skin surface contained the major portion of the applied dose, being 73.61, 75.024, 74.94, and 67.59% of total applied dose at the 8, 24, 72, and 168 hour groups, respectively. Mean total radiolabel recovery was 100.5, 95.8, 102.9, and 95.6% of total applied dose at the 8, 24, 72, and 168 hour sacrifice groups, respectively (Table KIIIA1 7.6.1/01-4).

Table KIIIA1 7.6.1/01-4: Recovery of total radioactivity up to 168 hours following a 8-hour topical exposure to a 0.1 g/L high-dilution formulation [0.975 to 0.992 µg a.c./cm^2 skin] of BYI02960 in SYP13529 (up to 168-hours post initiation of exposure)

	Data expressed as per cent of applied dose			
	Termination hour for group [N=4/group/timepoint]			
Absorption:	**8**	**24**	**72**	**168**
Directly absorbed dose				
Urine	0.078±0.048	0.572±0.277	1.823±0.450	3.004±0.274
Faeces	ND	0.109±0.094	0.622±0.203	0.443±0.254
Cage wash	ND	0.121±0.152	0.341±0.394	0.698±0.438
Cardiac blood	0.068±0.013	0.055±0.075	0.111±0.088	0.089±0.063
Non treated skin**[A]**	1.551±0.382	0.527±0.107	0.755±0.117	0.794±0.047
Carcass	2.625±1.446	1.399±0.183	1.625±0.466	3.093±0.690
Total directly absorbed	**4.323±1.676**	**2.783±0.724**	**5.278±0.736**	**8.120±1.019**
Absorbable dose				
Directly absorbed dose	**4.323±1.676**	**2.783±0.724**	**5.278±0.736**	**8.120±1.019**
Stratum corneum **[B]**	8.735±4.498	6.258±3.028	11.692±3.142	10.369±2.677
Treated skin **[C]**	2.049±0.789	0.867±0.563	0.828±0.534	1.355±1.192
Surrounding skin **[D]**	1.533±0.158	0.410±0.031	0.400±0.260	0.734±0.337
Total absorbable	**16.64±5.167**	**10.32±3.852**	**18.20±2.704**	**20.57±3.152**
Unabsorbed dose				
Skin swabs (8 hr)	73.613±3.267	75.024±4.616	74.941±4.023	67.591±2.435
Terminal swabs	NS	7.064±2.688	4.918±1.738	0.703±0.296
Surrounding swabs	4.373±3.020	0.354±0.460	0.672±0.531	0.372±0.157
Tape strips 1-2	3.500±1.555	2.610±1.334	3.661±0.779	3.664±0.836
Fur	1.710±0.830	NS	NS	NS
Dressings	0.638±0.867	0.414±0.282	0.490±0.246	2.652± 1.911
Total non-absorbed	**83.83±3.578**	**85.462±7.453**	**84.68±3.637**	**74.982±4.036**
Total recovered	**100.474±3.487**	**95.785±3.267**	**102.880±1.025**	**95.560±1.990**

ND = not detected. NS = no sample

Data excerpted from table on pages 12 and 28 of the study report.

'A' – denotes sum of radioactivity in skin distant from the site of test substance application. 'B' – denotes sum of radioactivity in tape strips excluding number 1 & 2 which were considered to be non-absorbed dose. 'C'– denotes sum of radioactivity found in treated skin excluding after tape-stripping procedure. 'D'– denotes sum of radioactivity in skin adjacent to the treated skin.

The study authors stated that as a conservative approach, radiolabel recovery in the skin compartment (stratum corneum, treated skin and surrounding skin) was regarded as

absorbable. They therefore calculated the amount of BYI 02960 potentially absorbable following an 8-hour exposure as ranging from 17.0 to 21.8% for the 200 g/L neat product, 5.54% to 9.66% for the 0.625 g/L intermediate dilution, and from 10.3% to 20.6% for the highly diluted 0.1 g/L formulation. They concluded that the upper values from these ranges could be regarded as the maximum potentially absorbed percentages.

The evaluator also agrees with the study authors decision to regard the fraction remaining in the stratum corneum, treated skin, and surrounding skin as being potentially absorbable dose, and notes that the last available layers of stratum corneum in 168 hour groups (tape strips 12 or 9 for the individuals with the highest absorption values) all contained radiolabel, albeit at levels 32 to 64% lower than that on the first (surface) tape strips (Table KIIIA1 7.6.1/01-5). This finding demonstrated that dermal absorption was potentially continuing up to and including the 168 hour timepoint.

Table KIIIA1 7.6.1/01-5**: Details from 168 hour sacrifice animals with the highest absorptions**

Compartment	Time (h)	Concentration (g/L) [(µg/cm^2]		
		200 [1920 to 2080]	0.625 [6.08 to 6.42]	0.1 [0.975 to 0.99]
		UM4M0016	UM8M0033	UM12M0046
urine	8	0.012	0.045	0.717
	24	0.261	0.294	0.854
	48	0.444	0.170	0.305
	72	0.247	0.145	0.225
	96	0.263	0.103	0.194
	120	0.755	0.087	0.171
	144	0.454	0.093	0.113
	168	0.198	0.079	0.210
faeces	8	ND	0.023	0.091
	24	0.051	0.063	ND
	48	0.306	0.074	0.241
	72	0.237	ND	ND
	96	0.275	ND	ND
	120	0.312	ND	0.104
	144	0.131	0.048	ND
	168	0.169	ND	0.172
cage wash	8	ND	0.08	0.525
	24	0.121	0.04	0.169
	48	0.295	0.033	ND
	72	0.711	0.022	ND
	96	3.407	0.032	ND
	120	0.436	0.064	0.091
	144	0.222	0.026	0.209
	168	0.172	ND	0.229
swab 8		53.302	74.851	63.522
swab x		0.001	0.079	0.489
swab y		0.004	0.059	0.318
swab z	8	0.004	0.082	0.588
swab 168	168	0.656	2.164	0.068
swab x		0.003	0.28	0.091
swab y		0.016	0.109	0.067
swab z		0.008	0.229	0.135
SC1		0.122	0.605	1.426
SC2		0.366	1.986	1.696
SC3		0.124	1.591	2.683
SC4		0.075	0.661	1.795
SC5		0.108	0.952	2.334
SC6		0.079	0.764	1.272
SC7		0.107	0.927	1.644
SC8		0.086	0.893	1.153
SC9		0.068	0.410	0.566
SC10		0.125	NS	NS
SC11		0.045	NS	NS
SC12		0.044	NS	NS
SC13		NS	NS	NS
SC14		NS	NS	NS
SC15		NS	NS	NS
SC16		NS	NS	NS
SC17		NS	NS	NS
SC18		NS	NS	NS
SC19		NS	NS	NS
SC20		NS	NS	NS
Cardiac blood		0.027	0.019	0.088
Treated skin		0.47	0.898	2.245
Non treated skin		4.765	0.177	0.74
Surrounding skin		13.415	0.104	0.623
Carcass		0.975	0.461	4.117
Surrounding swab		0.21	0.174	0.314
Dressing		12.891	5.379	1.944
Fur (dose site)		0.541	ns	NS
Total recovery		**98.114**	**95.374**	**94.538**

Compartment	Time (h)	Concentration (g/L) [(µg/cm^2]		
		200 [1920 to 2080]	0.625 [6.08 to 6.42]	0.1 [0.975 to 0.99]
		UM4M0016	UM8M0033	UM12M0046
potentially absorbable		**29.9**	**9.38**	**23.9**

Note: Radiolabel recovered from compartments ***bolded and italicised*** in the table above were not included in the calculation of the potentially absorbable fraction. 'ND' – denotes 'not detected'. 'NS' – denotes 'no sample'. SC = stratum corneum. Data excerpted from pages 57, 58, 66, 67, 75 and 76, with the exception of the potentially absorbable fraction, which was calculated by the evaluator using Microsoft Excel and rounded to three significant figures.

The evaluator also agrees with the study evaluator's conclusion to use values derived from the individual in each group with the highest levels of dermal absorption. This is consistent with advice provided in the OECD Guidance Notes on Dermal Absorption (2011), where the higher value of a range obtained in group sizes of four or less is recommended when inter-individual variation is high (SD equal to or greater than 25% of the mean). However, the subsequent values provided by the study evaluator were the highest group means over the four time points only. On an individual animal basis, the highest absorptions of test substance were calculated by the evaluator to be 29.9, 9.38, and 23.9% for the concentrate, 0.625 g/L, and 0.1 g/L dilutions, respectively at 168 hours (Table KIIIA1 7.6.1/01-5). The study evaluator concludes that these values better capture the potential intra-species dermal absorption variability for the test substance in rats *in vivo*.

III. CONCLUSIONS

Under the conditions of this *in vivo* dermal absorption study in male Wistar rats (4/dose) with a 200 g/L SL BYI02960 formulation, the highest individual potential dermal absorption over 168 hours for each concentration were 29.9%, 9.38%, and 23.9% at the three concentrations of 200 g/L, 0.625 g/L and 0.1 g/L, respectively.

The dermal absorption data from both *in vivo* and *in vitro* dermal absorption studies were used to estimate the potential *in vivo* dermal absorption factors for humans.

A summary of the overall dermal absorption of BYI02960 from the SYP13527 formuulation is presented in Table KIIIA1 7.6.1/01-X which includes the value of *in vitro* dermal absorption in rat and human skin (MRID 48844558).

The extrapolated human dermal absorption for BYI02960 200 g/L SL formulation is based on the maximum *in vivo* absorption in rats 168 hours following initiation of exposure and the comparative absorption *in vitro* in rat and human skin 24 hours post-exposure. The absorption value for 168 hours from the *in vivo* study was used as *in vivo* dermal absorption was not considered complete by 24-hours.

The estimated maximum human dermal absorption factors *in vivo* were 42.1, 3.32, and 17.2% for the concentrate, intermediate, and highly diluted formulation, respectively (Table KIIIA1 7.6.1/01-6). These estimations were calculated using the highest potential dermal absorption values of individuals in each dosing group; an approach chosen on the basis of high intragroup variability and group sizes of four individuals. ***However, US EPA secondary reviewer does not agree with the conclusion concerning the human dermal absorption factor because there are no data for the exposure duration beyond 24 hours in the in-vitro human and rat skin***

studies. Therefore, the US EPA determines that the appropriate estimate for the potential human dermal absorption factor to be 7.42 % based on the data on the 24-hour exposure period and most dilute administered concentration (0.1 g/l).

Table KIIIA1 7.6.1/01-6: Summary of dermal absorption of BYI02960 200 g/L SL (8 hours exposure)

Study	Sample time (hours)	Per cent absorbed			Reference
		200 g/L (concentrate)	0.625 g/L	0.1 g/L	
In vitro - rat skin	24	0.145	5.667	6.607	Odin-Feurtet (2010) MRID 48844558
In vitro - human skin	24	0.204	2.008	4.753	
In vivo - rat	8	21.78	9.26	16.64	Odin-Feurtet (2010) MRID 48844557
	24 $\bar{x}$	19.63	8.28	10.32	
	168 $\bar{x}$	19.18	5.534	20.57	
	168 **max**[b]	29.9	9.38	23.9	
Estimated % $\bar{x}$ human dermal absorption factor (24 hours *in vivo data*) [a]		27.6	2.93	7.42	
Estimated % $\bar{x}$ human dermal absorption factor (168 hours *in vivo data*) [a]		27.0	1.96	14.8	
Estimated % *maximum* human dermal absorption factor (terminal results, 168 hours *in vivo data*) [a,b]		**42.1**	**3.32**	**17.2**	

[a] human dermal absorption factor (% absorbed) values were estimated using the formula:
In vivo human absorption (% absorbed) ≅ (*in vitro* human % absorption × *in vivo* rat % absorbed)/ *in vitro* rat % absorption
The estimates were rounded to three significant figures by the evaluator.

[b] Using the highest *in vivo* dermal absorption values of the individuals in each group (*i.e.,* Animal No's UM4M0016, UM8M0033, and UM12M0046 for the concentrate, 0.625 g/L, and 0.1 g/L dilutions, respectively).

Primary review: Australia
Secondary Review: US EPA

IIIA 7.6.2 Comparative dermal absorption, *in vitro* using rat and human skin

Report: Odin-Feurtet M (2010) BYI 02960 (SL200) Comparative in vitro dermal absorption study using human and rat skin. Bayer SAS, Bayer CropScience 355, Rue Dostoievski, BP 153, 06903 Sophia Antipolis Cedex France. Laboratory Report No. SA 09254. Sponsor identification number: Lynx-PSI N°TXRVP029. 28 September 2010. MRID: 48844558. Unpublished.

Guidelines: OECD 428 (2004), OECD 28 (2004), ECC Sanco/222/2000 Rev.7 (2004)

Deviations: None

GLP: Yes. Signed statements of GLP Compliance, Data Confidentiality, and Quality Assurance were submitted in the study report.

Executive summary:

The dermal penetration of a BYI02960 200g/L soluble concentrate (SL) formulation was tested *in vitro* using rat (dorsal) and human (abdominal) skin mounted in a a glass flow-through diffusion cell system with an exposure area of 1 cm² maintained at 32±2ºC (MRID 48844558). The test substance was applied as either the undiluted 200 g/L SL formulation, or as 0.625 g/L or 0.1 g/L aqueous dilutions. Penetration and absorption were followed using [pyridinylmethyl-^{14}C]-BYI02960, which was uniformly blended into the formulations prior to application. The formulated products were applied at a rate of 10 µL/cm² to 2 groups of 6 skins per dose level per species with the exception of the 0.625 g/L groups which had only 5 skins for humans and 4 for rats. The achieved concentrations of BYI02960 applied per area of skin was approximately 1800 to 2000, 4.5 to 5.0, and 0.9 to 1.1 µg/cm² for the 200, 0.625 and 0.1 g/L formulations, respectively. The formulation concentrations and application rates were designed to mimic potential field-use exposures.The applied formulation remained in contact with the skins for 8 hours. Serial receptor fluid samples (2×50 µL) were collected hourly for the 24 hour duration of the study. The volume of receptor fluid was maintained by the replacement with a volume of fresh receptor fluid, equal to the total aliquot volume. At 8 hours, the skin surfaces of all groups were washed with sponge swabs and freshly prepared 1% v/v Tween 80 in phosphate buffered saline. At 24 hours, the skin samples were swabbed again, and stripped using Monaderm adhesive tape to remove non-absorbed surface test material (first two strips) and stratum corneum (strips three until a shiny appearance had been reached, indicating complete removal). Stripping involved applying the tape for five seconds before removing the strip against the direction of hair growth.

For the 200 g/L formulation, mean total recovery was 106.1 and 105.5% of that applied for humans and rats, respectively. As a percentage of total applied dose, the potentially absorbable fraction (receptor fluid plus tape-stripped skin) over 24 hours was 0.204 and 0.145% for human and rat skin, respectively. The total absorbable dose was therefore approx. 1.4-fold greater for human skin than for rat skin. Of the potentially absorbable fraction, directly absorbed dose (receptor fluid and receptor fluid terminal) only comprised 0.016±0.029% and 0.071±0.071% of the total applied for human and rat skin, respectively. Stratum corneum contained just over half

(0.103±0.120%) of the total absorbable fraction in humans, and just over a third (0.055±0.075%) of the total absorbable for rats. Washing the skin at eight hours removed >105% of the applied dose for both species, while tape strips one and two at 24 hours removed 0.128 and 0.132% of the applied dose for human and rat skin, respectively.

For the 0.625 g/L formulation, mean total recovery was 100.3 and 96.4% of that applied for humans and rats, respectively. As a percentage of total applied dose, the potentially absorbable fraction (receptor fluid plus tape-stripped skin) over 24 hours was 2.008 and 5.667% for human and rat skin, respectively. The total absorbable dose was therefore approx. 2.5-fold greater for rat skin than for human skin. As was found for the concentrate formulation, directly absorbed dose (receptor fluid and receptor fluid terminal) was a minor contributor to the potentially absorbable total, being 0.392±0.344% and 1.075±0.330% for human and rat skin, respectively. Stratum corneum again contained just over half of the total absorbable fraction for both humans (1.149±0.534%) and rats (2.852±3.832%). Washing the skin at eight hours removed a mean of 97% and 85.5% of the applied dose for human and rat skin samples, respectively. Tape strips one and two at 24 hours removed 0.492 and 2.137% of the applied dose for human and rat skin, respectively.

For the 0.1 g/L formulation, mean total recovery was 106.1 and 105.5% of that applied for humans and rats, respectively. As a percentage of total applied dose, the potentially absorbable fraction (receptor fluid plus tape-stripped skin) over 24 hours was 4.753±2.963% and 6.607±2.796% for human and rat skin, respectively. The extent of interspecies difference at this concentration was therefore similar in magnitude to that for the concentrate, but in reverse direction, being only 1.4-fold higher for rat skin than for human skin. As was found for the two higher concentration levels, directly absorbed dose (receptor fluid and receptor fluid terminal) was a minor contributor to the potentially absorbable total, being 0.646±0.482% and 1.105±0.436% for human and rat skin, respectively. Stratum corneum again contained just over half of the total absorbable fraction for humans (2.998±1.828%) but was even higher for rats, comprising approx. two-thirds (4.409±2.893%) of the total. Washing the skin at eight hours removed a mean of 89.4% and 85.5% of the applied dose for human and rat skin samples, respectively. Tape strips one and two at 24 hours removed 0.99% and 5.86% of the applied dose for human and rat skin, respectively.

As penetration into the receptor chamber remained ongoing at the 24 hour termination point of the study for the majority of rat and human skin samples for the three formulation concentrations, test substance remaining in the stratum corneum were considered to be potentially absorbable.

Group mean values were used in the calculations of absorbable dose. Although standard deviations for the potentially absorbable fractions in all groups ranged from 42% (rat 0.1 g/L sample) to 106% (human 200 g/L sample) of the means, the variations were considered likely to be outliers arising from experimental variation/errors rather than representations of true biological variation.

Under the conditions of this *in vitro* dermal absorption study, the study author calculated the mean total potentially absorbable values in humans/rat skin samples,

respectively, over 24 hours to be 0.20/0.15% for the 200 g/L formulation, 2.0/5.7% for the 0.625 g/L formulation, and 4.8/6.6% for the 0.1 g/L formulation. The evaluator agreed with the study author's conclusions.

The results obtained in this study, using an *in vitro* flow-through diffusion cell system, demonstrate that penetration and absorption of BYI02960 from the 200 g/L SL formulation was relatively equivalent to within 30 to 40% ranges in rat and human skin when applied as the undiluted concentrate or highly diluted (0.1 g/L) formulations, but that dermal absorption was nearly 3-fold higher in rats for the intermediate dilution (0.625 g/L).

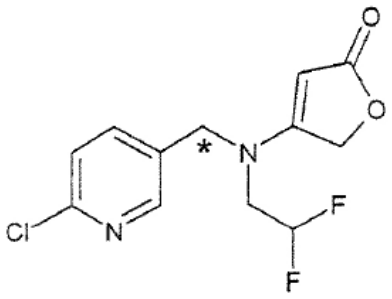
The *in vitro* dermal absorption data on isolated rat and human skin in combination with the *in vivo* rat dermal absorption data were used by the primary study evaluator to estimate the *in vivo* human dermal absorption factor; the factors were 42.1, 3.32, and 17.2% for the concentrate, intermediate, and highly diluted formulation, respectively. *However, US EPA secondary reviewer does not agree with the conclusion concerning the human dermal absorption factor because there are no data for the exposure duration beyond 24 hours in the in-vitro human and rat skin studies. Therefore, the US EPA determines that the appropriate estimate for the potential human dermal absorption factor to be 7.42 % based on the data on the 24-hour exposure period and most dilute administered concentration (0.1 g/l).*

This study is reliable (Acceptable) and fulfils the requirements of the OECD guideline for *in vitro* dermal absorption studies in rats and humans (OECD 428 (2004)).

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	BYI02960, as a component of a 200 g/L SL formulation.
	Source:	Bayer CropScience AG, Product Technology-Analytics Frankfurt, Frankfurt am Main, DEU.
	Lot/Batch #:	Ref material: Lot# NLL 7780-47-4
	Purity:	Ref material: 99.4% w/w (MS, ^{1}H-NMR, ^{19}F-NMR, UV); Analysed on 27 October 2008.
	Description:	White powder
	CAS #:	Not reported.
	Stability of test compound:	Stated to be stable for two years at 5±5°C.
2.	Radiolabel test material:	^{14}C- BYI 02960 technical ('BYI 02960*')



* Denotes position of the radiolabel. [pyridinylmethyl-^{14}C]

	Lot/Batch #:	ID# BECH 2123
	Radiochemical purity:	99% (HPLC, radioactivity-detector) >99% (HPLC, UV-detector 210 nm) >98% (TLC, scan) >98% for SL200 g/L and subsequent dilutions (RP HPLC method)
	Specific activity:	4.37 MBq/mg (0.0185 MBq/mg after radio dilution to 200 g/L)
	Measured concentration of radioactivity:	3.54 Mbq/mL (200 g/L SL), 2.76 Mbq/mL (0.625 g/L), 0.438 MBq/mL (0.1 g/L)
	Description:	Solid
	Stability of test compound:	The test materials were stable when stored as solution in appropriate solvent (e.g., acetonitrile) and stored at -18°C.
3.	Formulation blank:	The blank formulation (Batch ID# 2009-000409), which was devoid of BYI 02960, was blended with the non-labeled and radiolabeled ingredients to produce the undiluted concentrated 200 g/L SL formulation. The blank formulation was stored at -18°C.
4.	Vehicle for dilution	Water was used for dilution of 200 g/L SL formulation to 0.625 g/L and

5. Rat skin: Dermatomed dorsal skin from ten male Wistar Rj:WI (IOPS HAN) rats (age unspecified). Rats were sacrificed by cervical dislocation and the skin clipped, excised, dermatomed to thicknesses of 460-540 µm, and stored at -20°C until use.
6. Human skin: Dermatomed abdominal skin from seven female donors (aged unspecified), sample thickness range of 437 to 592 µm, from Biopredic, Rennes, France, stored at approximately -20°C until prepared for use.
7. Test substance concentrations: See Table KIIIA1 7.6.2/01-1

Table KIIIA1 7.6.2/01-1: Summary of the formulation, target concentration and skin dose

Formulation	Density (g/mL)	Nominal concentration (g/L)	Radiolabel concentration (MBq/mL)		Skin dose (µg ai/cm^2)	
			Nominal	achieved	nominal	achieved
BYI02960 (concentrate)	1.138	200	3.7	3.54	2000	1800 to 2000
BYI02960 (intermediate dilution)	1.00*	0.625	2.73	2.76	6.25	4.5 to 5.0
BYI02960 (high dilution)	1.00*	0.1	0.437	0.438	1	0.9 to 1.1

*Estimated. The study authors used achieved concentrations to estimate the dose recovery percentage for the 0.625 g/L formulation, but used nominal (theoretical) concentrations for the concentrate (200 g/L) and high (0.1 g/L) dilution. Data excerpted from pages 21, and 72 to 75 of the study report.

B. STUDY DESIGN AND METHODS

1. Experimental start and completion dates

   26 March 2010 to 10 September 2010

2. Dermal penetration and absorption assay

   The dermal penetration and absorption of BYI02960 as a 200 g/L component of SYP13527 was measured *in vitro* with rat and human skin. Frozen samples of dermatomed rat and human skin of 460-540 µm thickness for rats (mean thickness of 513, 520, and 522 for the 1, 6.25, and 2000 µg ai/cm^2 dosing groups) and 437 to 592 µm thickness for humans (mean thickness of 501, 502, and 503 for the 1, 6.25, and 2000 µg ai/cm^2 dosing groups) were mounted over the receptor chamber of a glass flow-through diffusion cell system (Franz's cell modified, CDL, Marseille, France). The stratum corneum faced upwards and a total exposed surface area of 1 cm^2 in diameter. The receptor chamber was then filled with receptor fluid (3 mL Eagle's medium supplemented with 5% bovine serum albumin, and 50 mg/L gentamycin; pH 7.3-7.4), and maintained at approximately 32±2°C for the experiment duration. To aid dissolution of test substance, receptor fluid was pumped through the receptor chamber at 1.5 mL/hour, and stirred by a magnetic bar for the eight hour exposure phase. The receptor fluid passing through the receptor chamber was

collected in glass vials held in a fraction collector, with samples collected hourly for the 24 hour duration of the study. Prior to use, the integrity of each skin membrane was assessed by the trans epidermal water loss (TWEL) method. Membranes with a water loss rate of <15 g/hm^2 (for both rats and humans) were considered intact and retained for use, in accordance with the in-house acceptable cut-off limit. All membranes were found to easily meet this criteria (TEWL range of 1.05 to 5.33 g/hm^2). Loss of water above the skin membranes was measured by an evaporimeter probe (Tewameter TM300®, System, Courage & Khazaba) secured over the donor chamber.

Preliminary studies using a test substance amount equal to that required to achieve the planned maximum concentration (1800 to 2000 µg/cm^2 skin) was added to 3 mL of receptor medium and incubated for at least 24 hours at 32˚C confirmed sufficient solubility up to 800 µg test substance/mL receptor fluid. On confirmation of appropriate solubility of test substance to avoid risks of back diffusion through skin membranes, a 10 µL volume of the test substance containing [^{14}C]-BYI02960 as either a 200 g/L component of SYP13527 or in a further dilution with water to 0.625 or 0.1 g/L, were applied to prepared skin membranes over a 1 cm^2 surface area. The formulation concentrations were intended to mimic those handled by occupational users during mixing/loading (concentrate), and spray application (dilutions). Dose levels of applied radiolabelled samples were confirmed for homogeneity and applied concentration by liquid scintillation counting (LSC) analysis before, during, and after dosing.

The achieved concentration was used to calculate the dose recovery percentage for the 0.625 g/L formulation, whereas the nominal dose was used for such calculations of the concentrate and high dilution formulations (page 44 of the study report). The basis for not using achieved doses in the latter groups was not reported.

The applied formulation remained in contact with the skin samples for eight hours, at which time the exposed surface of all skin samples was washed with sponge swabs and freshly prepared 1% (v/v) Tween 80 in phosphate buffered saline. The membranes were maintained for a further 16 hours (study hour 24), where upon they were swabbed again, and stripped using Monaderm adhesive tape to remove non-absorbed surface test material (first two strips) and stratum corneum (strips three until a shiny appearance had been reached, indicating complete removal). Stripping involved applying the tape for five seconds before removing the strip against the direction of hair growth. The skin swabs at 8 (with separation of the last three swabs taken) and 24 hours were solubilised in specified volumes of Soluene®. Tape strips were individually solubilised in tetrahydyrofuran. Skin membranes and surrounding skin were separately solubilised in Soluene®. Scintillation fluid was added directly to all solubilised samples and they were then subjected to LSC analysis. Diffusion cell components including the donor and receptor chambers, stirring bar and tubing, were separately soaked for 12 hours in a 50:50 v/v acetronitrile/distilled water formulation after disassembly at study termination, the washings were weighed and duplicate weighed aliquots were

then subjected to LSC analysis. Remaining diffusion cell components were monitored for residual radioactivity using a Geiger-Muller monitor, and if any activity was detected the component area was swabbed until no further signal was obtained. The receptor fluid (interim samples at 2, 12 and 22 hours) and receptor fluid terminal (final total volume of receptor fluid at study termination) were analysed by LSC. Samples were either subjected to LSC analysis at the time of collection or were stored at -20°C in the dark until required.

LSC analysis proceeded by counting radioactivity for ten minutes or for 2 sigma % using a Packard 1900 TR counter. The scintillation counter was recalibrated when a deviation greater than 2% was observed when counting quality control standards. The limit of detection was defined as 2-fold higher radioactivity levels than that measured in blank samples. Calculation of dose recovery was undertaken using the Debra 5.2a software and Microsoft Excel. In some instances, rounding created differences between means and precise individual values.

Results expressed in terms of flux were not provided by the study authors and were not calculated by the evaluator.

II. RESULTS AND DISCUSSION

Upon analysis, the study authors noted unacceptably high inter-individual variability in absorption results for the initial six rat membranes (R13 to R18) exposed to the 1 µg ai/cm^2 dose, and excluded these results from further analysis. Results from samples R12 (6.25 µg ai/cm^2 dose), H15 and R19 (1.25 µg ai/cm^2 doses), were excluded based on a 'technical problem at swabbing', and those from H09 and R08 (6.25 µg ai/cm^2 doses) were excluded on the basis of being outliers ('different direct absorption compared to other cells in the group').

Directly absorbed dose was defined by the study authors as radiolabel recovery from the receptor fluid, receptor fluid terminal, and receptor chamber over 24 hours. Potentially absorbed dose was defined as radiolabel in stratum corneum (excluding tape strips 1 and 2) and within residual skin after tape stripping, together with the directly absorbed dose.

The calculated maximum concentration of BYI 02960 in the receptor fluid was 0.307 µg/mL, at least 2606-fold less than the demonstrated solubility of the test substance of up to and including 800 µg/mL. Therefore, there was therefore no risk of the test substance back-diffusing into the donor chamber.

For all dosing concentrations, the majority (85.4% to 105.6%) of applied radiolabel was recovered in swabs of the exposed skin at eight hours. There was no radiolabel detected in the receptor chamber component of the diffusion cell at any of the concentrations tested.

A. 200 g/L BYI 02960 CONCENTRATE

Total mean radiolabel recovery for 200 g/L [^{14}C]-BYI 02960 concentrate formulation was 106.1 and 105.5% of that applied for humans and rats, respectively. As a percentage of total applied dose, the potentially absorbable fraction (receptor fluid plus tape-stripped skin) over 24 hours was 0.204 and 0.145% for human and rat skin, respectively (Table KIIIA1 7.6.2/01-2). The total absorbable dose was therefore approx. 1.4-fold greater for human skin than for rat skin. Of the potentially absorbable fraction, directly absorbed dose (receptor fluid and receptor fluid terminal) only comprised 0.016±0.029% and 0.071±0.071% of the total applied for human and rat skin, respectively. Stratum corneum contained just over half (0.103±0.120%) of the total absorbable fraction in humans, and just over a third (0.055±0.075%) of the total absorbable for rats. Washing the skin at eight hours removed >105% of the applied dose for both species, while tape strips one and two (unabsorbable dose) at 24 hours removed 0.128 and 0.132% of the applied dose for human and rat skin, respectively.

Table KIIIA1 7.6.2/01-2: Recovery of total radioactivity at 24 hours following a 8-hour topical exposure to a 200 g/L undiluted concentrate [1800 to 2000 µg a.c./cm^2 skin] of BYI02960 in SYP13527 (16-hours after end of exposure)

	Data expressed as a per cent of applied dose	
	Human [N=6]	**Rat [N=6]**
Directly absorbed dose		
Receptor fluid	0.014±0.024	0.071±0.071
Receptor fluid terminal	0.002±0.005	ND
Total directly absorbed **[A]**	0.016±0.029	0.071±0.071
Absorbable dose		
Directly absorbed dose	0.016±0.029	0.071±0.071
Stratum corneum **[B]**	0.103±0.120	0.055±0.075
Skin **[C]**	0.085±0.087	0.019±0.015
Total absorbable [D]	**0.204±0.218**	**0.145±0.091**
Unabsorbed dose		
Skin swabs (8 hours)	105.611±4.373	105.099±1.413
Skin swabs (24 hours)**[E]**	0.087±0.146	0.015±0.012
Donor chamber	0.074±0.106	0.101±0.248
Tape strips 1 & 2	0.128±0.131	0.132±0.214
Total non-absorbed	105.900±4.495	105.347±1.519
Total recovered	**106.104±4.648**	**105.492±1.544**

ND = not detected Data excerpted from table on page 23 of the study report.

'A' – sum of radioactivity found in receptor fluid (0-24h), receptor fluid terminal and receptor chamber. 'B' – tape strips excluding number 1 & 2 which were considered to be non-absorbed dose. 'C' – denotes sum of radioactivity found in skin after tape-stripping procedure and in surrounding skin. 'D' – sum of radioactivity in Total % directly absorbed and Total % of dose site. 'E' – denotes sum of radioactivity found in swabs at termination and in surrounding swabs.

Penetration kinetics data for the fractions in the receptor fluid showed that dermal absorption of the 200 g/L formulation remained in progress for at least 4 of the 6 rat skin samples at 24 hours (Figure KIIIA1 7.6.2/01-A). Data was not provided for human samples based on a very low penetration.

Figure KIIIA1 7.6.2/01-A: Penetration kinetics (cumulative recovery) in rat skin of a 200 g/L undiluted concentrate [approx. 2000 µg a.c./cm^2 skin] of BYI02960 in SYP13527, 0-22.5 hours (8-hour exposure):

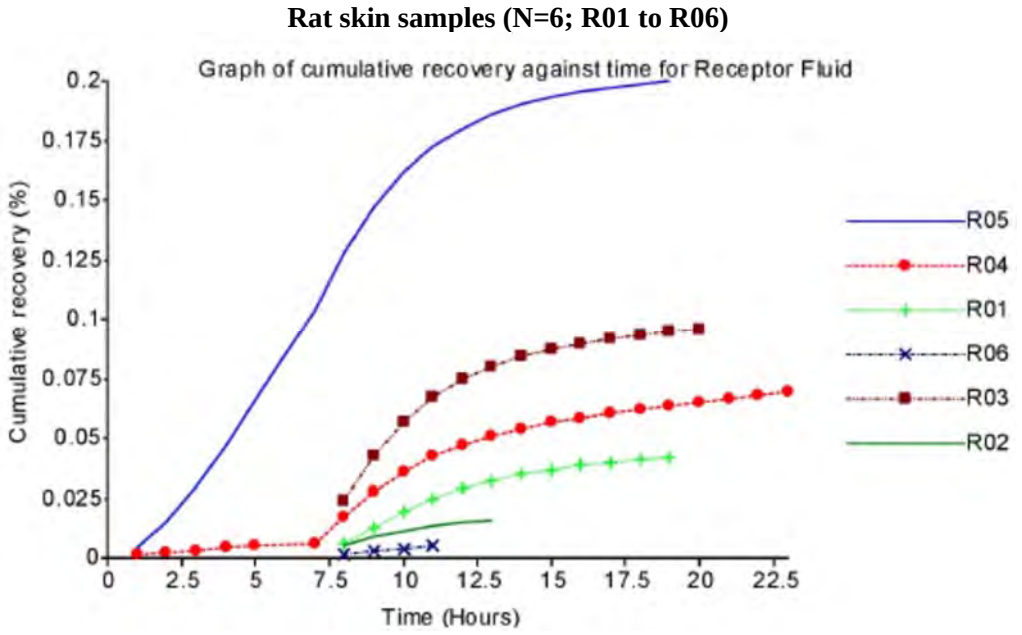


Figure excerpted from page 31 of the study report.

B. 0.625 g/L BYI 02960 AQUEOUS DILUTION

Total mean radiolabel recovery for 0.625 g/L [^{14}C]-BYI 02960 intermediate dilution formulation was 100.3 and 96.4% of that applied for humans and rats, respectively. As a percentage of total applied dose, the potentially absorbable fraction (receptor fluid plus tape-stripped skin) over 24 hours was 2.008 and 5.667% for human and rat skin, respectively (Table KIIIA1 7.6.2/01-3). The total absorbable dose was therefore approx. 3-fold greater for rat skin than for human skin. As was found for the concentrate formulation, directly absorbed dose (receptor fluid and receptor fluid terminal) was a minor contributor to the potentially absorbable total, being 0.392±0.344% and 1.075±0.330% for human and rat skin, respectively. Stratum corneum again contained just over half of the total absorbable fraction for both humans (1.149±0.534%) and rats (2.852±3.832%). Washing the skin at eight hours removed a mean of 97% and 85.5% of the applied dose for human and rat skin samples, respectively. Tape strips one and two (unabsorbable dose) at 24 hours removed 0.492 and 2.137% of the applied dose for human and rat skin, respectively.

Table KIIIA1 7.6.2/01-3. Recovery of total radioactivity at 24 hours following a 8-hour topical exposure to a 0.625 g/L dilution [4.5 to 5 µg a.c./cm^2 skin] of BYI02960 in SYP13527 (16-hours after end of exposure)

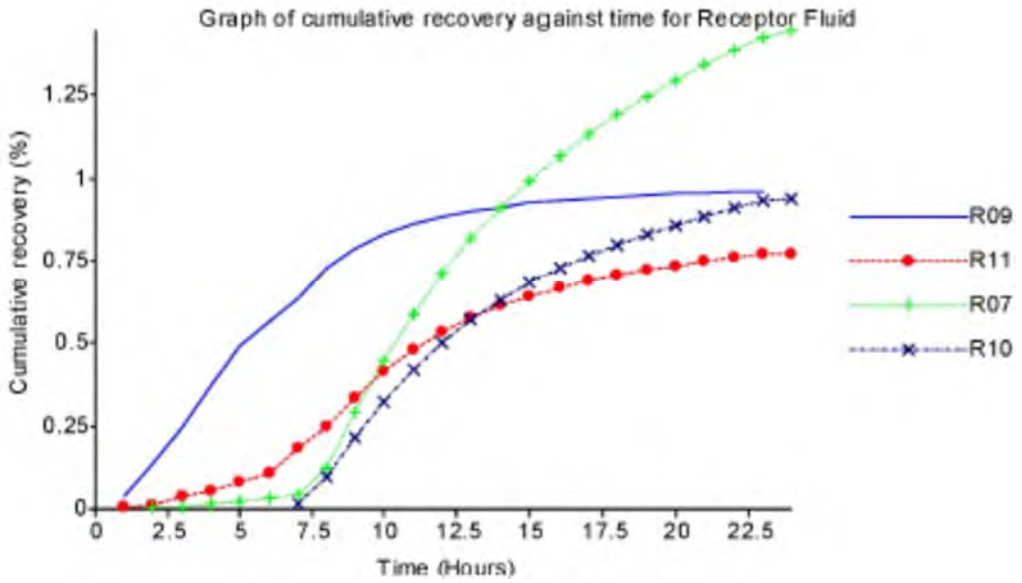
	Data expressed as a per cent of applied dose	
	Human [N=5]	**Rat [N=4]**
Directly absorbed dose		
Receptor fluid	0.375±0.321	1.029±0.290
▪ Receptor fluid terminal	0.017±0.024	0.046±0.046
Total directly absorbed **[A]**	0.392±0.344	1.075±0.330
Absorbable dose		
Directly absorbed dose	0.392±0.344	1.075±0.330
▪ Stratum corneum **[B]**	1.149±0.534	2.852±3.832
▪ Skin **[C]**	0.467±0.323	1.740±1.860
▪ **Total absorbable [D]**	**2.008±1.073**	**5.667±5.961**
Unabsorbed dose		
Skin swabs (8 hours)	97.282±1.723	85.495±6.246
▪ Skin swabs (24 hours)**[E]**	0.442±0.306	0.629±0.662
Donor chamber	0.086±0.080	2.429±2.609
Tape strips 1 to 2	0.492±0.256	2.137±1.170
Total non-absorbed	98.303±1.666	90.690±3.986
Total recovered	**100.311±1.896**	**96.357±5.984**

Data excerpted from table on page 23 of the study report. 'A' – sum of radioactivity found in receptor fluid (0-24h), receptor fluid terminal and receptor chamber. 'B' – tape strips excluding number 1 & 2 which were considered to be non-absorbed dose. 'C' – denotes sum of radioactivity found in skin after tape-stripping procedure and in surrounding skin. 'D' – sum of radioactivity in Total % directly absorbed and Total % of dose site. 'E' – denotes sum of radioactivity found in swabs at termination and in surrounding swabs.

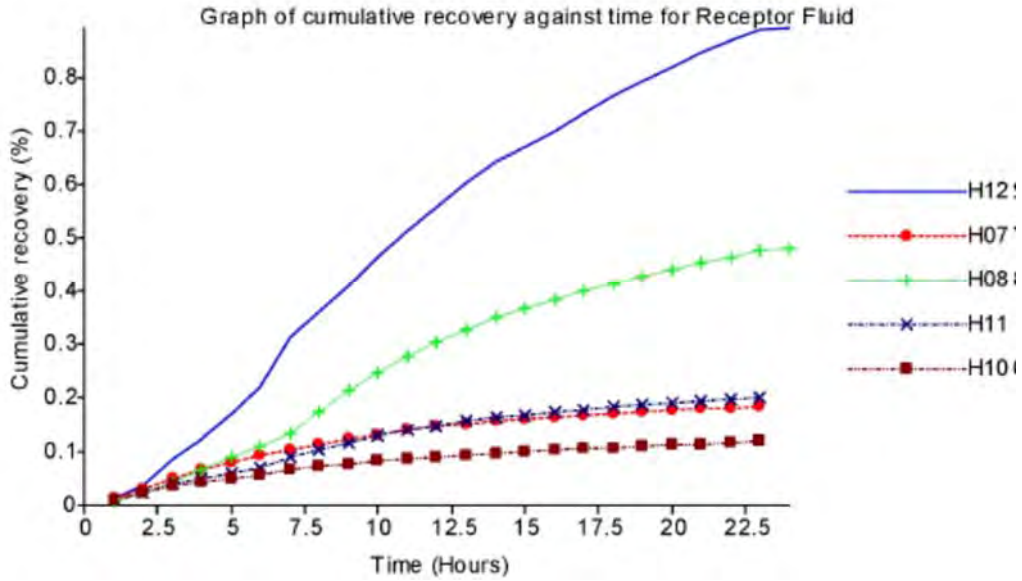
Penetration kinetics data for the fractions in the receptor fluid showed that dermal absorption of the 0.625 g/L formulation remained in progress for two of the four rat skin samples and two of the five human samples at 24 hours (Figure KIIIA1 7.6.2/01-B).

Figure KIIIA1 7.6.2/01-B: Penetration kinetics (cumulative recovery) in rat and human skin of a 0.625 g/L dilution [4.5 to 5 µg a.c./cm^2 skin] of BYI02960 in SYP13527; 0–22.5 hours (8-hour exposure):

Rat skin (N=4; R07, 9, 10, 11)



Human skin (N=5; H07, 8, 10, 11, 12)



Figures excerpted from pages 32 and 33 of the study report.

C. 0.1 g/L BYI 02960 AQUEOUS DILUTION

Total mean radiolabel recovery for 0.1 g/L [^{14}C]-BYI 02960 high-dilution formulation was 106.1 and 105.5% of that applied for humans and rats, respectively. As a percentage of total applied dose, the potentially absorbable fraction (receptor fluid plus tape-stripped skin) over 24 hours was 4.753±2.963% and 6.607±2.796% for human and rat skin, respectively (Table KIIIA1 7.6.2/01-4). The extent of interspecies difference at this concentration was therefore similar in magnitude to that for the concentrate, but in reverse direction, being only 1.4-fold higher for rat skin than for human skin. As was found for the two higher concentration levels, directly absorbed dose (receptor fluid and receptor fluid terminal) was a minor contributor to the total potentially absorbable, being 0.646±0.482% and 1.105±0.436% for human and rat skin, respectively. Stratum corneum again contained just over half of the total absorbable fraction for humans (2.998±1.828%) but was even higher for rats, comprising approx. two-thirds (4.409±2.893%) of the total. Washing the skin at eight hours removed a mean of 89.4% and 85.5% of the applied dose for human and rat skin samples, respectively. Tape strips one and two (unabsorbable dose) at 24 hours removed 0.99% and 5.86% of the applied dose for human and rat skin, respectively.

Table KIIIA1 7.6.2/01-4: Recovery of total radioactivity at 24 hours following a 8-hour topical exposure to a 0.1 g/L dilution [0.9 to 1.1 µg a.c./cm^2 skin] of BYI02960 in SYP13527 (16-hours after end of exposure)

	Data expressed as a per cent of applied dose	
	Human [N=6]	**Rat [N=6]**
Directly absorbed dose		
Receptor fluid	0.610±0.432	1.021±0.425
▪ Receptor fluid terminal	0.037±0.051	0.084±0.056
Total directly absorbed **[A]**	**0.646±0.482**	**1.105±0.436**
Absorbable dose		
Directly absorbed dose	0.646±0.482	1.105±0.436
▪ Stratum corneum **[B]**	2.998±1.828	4.409±2.893
▪ Skin **[C]**	1.108±0.905	1.093±0.856
▪ **Total absorbable [D]**	**4.753±2.963**	**6.607±2.796**
Unabsorbed dose		
Skin swabs (8 hours)	89.404±3.416	85.475±8.399
▪ Skin swabs (24 hours)**[E]**	0.966±0.888	5.658±3.189
Donor chamber	0.100±0.223	ND
Tape strips 1 to 2	0.993±0.515	5.859±3.497
Total non-absorbed	91.463±3.449	96.992±4.238
Total recovered	**106.104±4.648**	**105.492±1.544**

ND = not detected

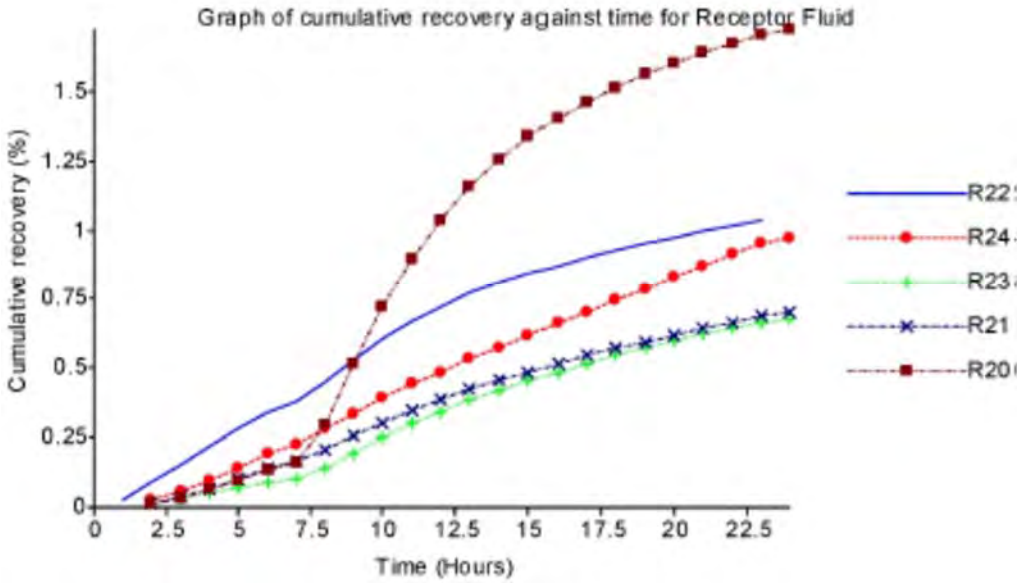
Data excerpted from table on page 23 of the study report.

'A' – sum of radioactivity found in receptor fluid (0-24h), receptor fluid terminal and receptor chamber. 'B' – tape strips excluding number 1 & 2 which were considered to be non-absorbed dose. 'C' – denotes sum of radioactivity found in skin after tape-stripping procedure and in surrounding skin. 'D' – sum of radioactivity in Total % directly absorbed and Total % of dose site. 'E' – denotes sum of radioactivity found in swabs at termination and in surrounding swabs.

Penetration kinetics data for the fractions in the receptor fluid showed that dermal absorption of the 0.1 g/L formulation remained in progress for all five of the rat skin samples and three of the five human samples at 24 hours (Figure KIIIA1 7.6.2/01-C).

Figure KIIIA1 7.6.2/01-C: Penetration kinetics (cumulative recovery) in rat skin of a 0.1 g/L dilution [1800 to 2000 µg a.c./cm^2 skin] of BYI02960 in SYP13527; 0–22.5 hours (8-hour exposure):

Rat skin (N=5; R20 to R24)



Human skin (N=5; H13, 14, 16, 17, 18)

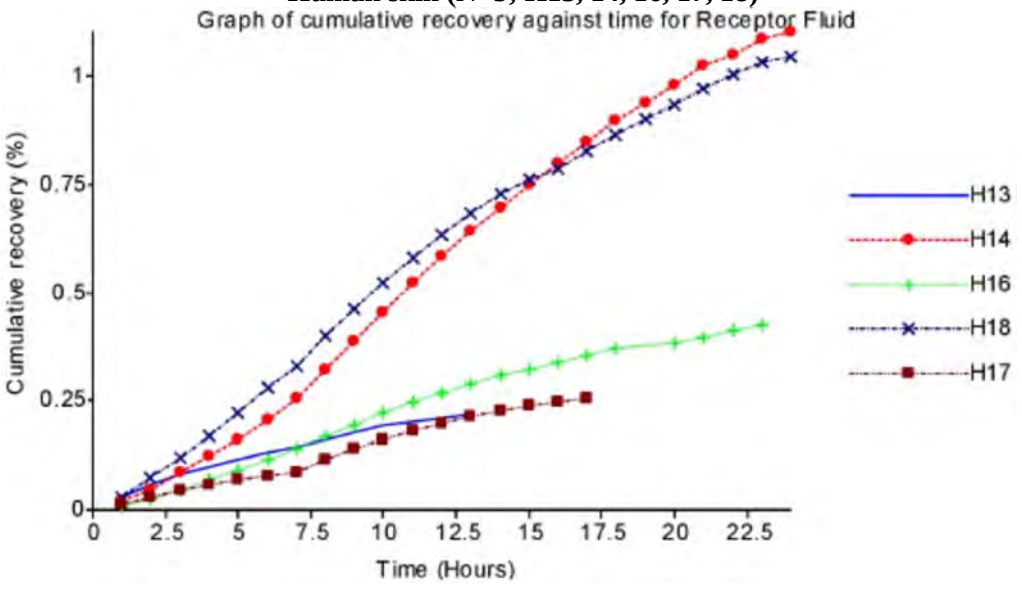


Figure excerpted from pages 34 and 35 of the study report.

The study authors regarded that for human/rat *in vitro* skin samples, respectively, the mean total potentially absorbable values over 24 hours were 0.20/0.15% for the 200 g/L formulation, 2.0/5.7% for the 0.625 g/L formulation, and 4.8/6.6% for the 0.1 g/L formulation.

The evaluator notes that as penetration into the receptor chamber remained ongoing at the 24 hour termination point of the study for the majority of rat and human skin samples for the three formulation concentrations, test substance remaining in the stratum corneum should be considered as potentially absorbable.

The evaluator also notes that the standard deviations for the potentially absorbable fractions in all groups ranged from 42% (rat 0.1 g/L sample) to 106% (human 200 g/L sample) of the means. The OECD Guidance Notes (2011) recommend that inter-individual variation is high when the standard deviation exceeds 25% of the mean for group sizes of four or less, and consideration should be given to using upper range from each group in these cases. Of the groups, only one contained four animals or less (rat 0.625 g/L) sample and the standard deviation in this group was 105% of the mean. However, based on the technical challenges of *in vitro* dermal absorption studies, the evaluator considers the variations in this study to be more likely to be outliers arising from experimental variation/errors rather than representations of true biological variation. For these reasons, the evaluator agrees with the study author's use of group mean values for this *in vitro* study, rather than using those from the individual sample with the highest dermal absorption of each group.

The evaluator therefore agrees with the study authors conclusions on the potentially absorbable fractions of the 200 g/L and dilute formulations of BYI 02960 (in SYP13527) in human and rat skin *in vitro*.

III. CONCLUSIONS

Under the conditions of this *in vitro* dermal absorption study, the mean total potentially absorbable values humans/rat skin samples, respectively, over 24 hours were 0.20/0.15% for the 200 g/L formulation, 2.0/5.7% for the 0.625 g/L formulation, and 4.8/6.6% for the 0.1 g/L formulation.

The results obtained in this study, using an *in vitro* flow-through diffusion cell system, demonstrate that penetration and absorption of BYI02960 from the 200 g/L SL formulation was similar in magnitude to that for the highly diluted (0.1 g/L) formulation albeit in reverse direction, but that dermal absorption was nearly 3-fold higher in rats for the intermediate dilution (0.625 g/L).

A summary of the overall dermal absorption of BYI02960 from the SYP13527 formuulation is presented in Table KIIIA1 7.6.2/01-5 which includes the value of *in vivo* dermal absorption in rats (MRID 48844557). From these data, a human dermal absorption factor can be estimated.

The extrapolated human dermal absorption for BYI02960 200 g/L SL formulation is based on the maximum *in vivo* absorption in rats 168 hours following initiation of

exposure and the comparative absorption *in vitro* in rat and human skin 24 hours post-exposure. The absorption value for 168 hours from the *in vivo* study was used as *in vivo* dermal absorption was not considered complete by 24-hours. **The primary evaluator's estimated human dermal absorption factors are 42.1, 3.32, and 17.2% for the concentrate, intermediate, and highly diluted formulation, respectively (Table KIIIA1 7.6.2/01-5).**

For information purposes, the extrapolated human dermal absorption using 24-hour data from both the *in vitro* and *in vivo* studies have also been presented.

Neither the study author (Odin-Feurtet) nor the applicant provided an estimate of the potential *in vivo* dermal absorption for humans.

Table KIIIA1 7.6.2/01-5: Summary of dermal absorption of BYI02960 200 g/L SL (8 hours exposure)

Study	Sample time (hours)	Per cent absorbed			Reference
		200 g/L (concentrate)	0.625 g/L	0.1 g/L	
In vitro - rat skin	24	0.145	5.667	6.607	Odin-Feurtet (2010) MRID
In vitro - human skin	24	0.204	2.008	4.753	
In vivo - rat	8	21.78	9.26	16.64	Odin-Feurtet (2010) MRID 48844557
	24 $\bar{x}$	19.63	8.28	10.32	
	168 $\bar{x}$	19.18	5.534	20.57	
	168 **max**[b]	29.9	9.38	23.9	
Estimated % $\bar{x}$ human dermal absorption factor (24 hours *in vivo data*) [a]		**27.6**	**2.93**	**7.42**	
Estimated % $\bar{x}$ human dermal absorption factor (168 hours *in vivo data*) [a]		27.0	1.96	14.8	
Estimated % *maximum* human dermal absorption factor (terminal results, 168 hours *in vivo data*) [a,b]		**42.1**	**3.32**	**17.2**	

[a] human dermal absorption factor (% absorbed) values were estimated using the formula: *In vivo* human absorption (% absorbed) ≅ (*in vitro* human % absorption × *in vivo* rat % absorbed)/ *in vitro* rat % absorption

[b] Using the highest *in vivo* dermal absorption values of the individuals in each group (*i.e.*, Animal No's UM4M0016, UM8M0033, and UM12M0046 for the concentrate, 0.625 g/L, and 0.1 g/L dilutions, respectively).

However, US EPA secondary reviewer does not agree with the conclusion concerning the human dermal absorption factor because there are no data for the exposure duration beyond 24 hours in the in-vitro human and rat skin studies. Therefore, the US EPA determines that the appropriate estimate for the potential human dermal absorption factor to be 7.42 % based on the data on the 24-hour exposure period and most dilute administered concentration (0.1 g/l).

Primary review: Australia
Secondary review: US EPA & PMRA

B.6.8.3/08 *In vitro* genotoxicity—Bacterial Assay for Gene Mutation

Report:	Mochizuki, N. and Kanaguchi, Y. (1997). IM-0: Reverse Mutation Study on Bacteria. Nippon Soda Co., Ltd, Toxicology Laboratory, Odawara Research Center, Kanagawa, Japan. Study ID C007426. Lab Project ID: G-949. Ref: M-195904-01-2. MRID 44988432. Unpublished.
Dates of work:	19 November – 6 December 1993; Final report 6 June 1994. 1st amendment 30 September 1997.
Guidelines:	EPA Guideline 84-2 (1984), OECD No. 471 (1983), JMAFF 59 Nohsan No. 4200 (1985).
GLP/QA:	Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary:

In independent trials of a reverse gene mutation assay, (6-chloro-3-pyridyl)methanol (IM-0) (99.14% Lot No. NK-3120) was evaluated for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2*uvrA* with and without an exogenous metabolic activation system (5,6-benzoflavone-induced rat liver S9) in two phases using the pre-incubation method (Experiment 1 and 2). The dose levels tested were 0, 313, 625, 1250, 2500 and 5000 µg/plate. The test substance was administered to the test system as a solution in dimethyl sulfoxide (DMSO).

Slight precipitation was observed at the limit dose of 5000 µg/plate in Experiment 1, but not Experiment 2. The positive and vehicle controls fulfilled the requirements for a valid test. In the mutagenicity assay no positive mutagenic response occurred.

Under the conditions of this study, IM-0 was tested up to the limit dose and was negative for mutagenic activity in non-activated and S9-activated test systems.

The study is classified as **reliable (acceptable/guideline)** and satisfies the guideline requirements (OECD 471 and equivalents) for *in vitro* mutagenicity (bacterial reverse gene mutation) data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: IM-0 (synonym: CPA; [6-chloro-3-pyridyl]methanol)
   Lot/Batch #: NK-3120
   Purity: 99.14%
   Description: White crystal
   CAS # 21543-49-7
   Stability of test compound: Stability analysis indicated that the test material was soluble (≥ 50 mg/mL in water and DMSO) and stable for 4 hours at 24°C in 5 % aqueous solutions.
2. Control materials
   Solvent control: Dimethyl sulfoxide (DMSO) at 100 µL/plate for all materials

Positive control, non-activation:

Positive control	Dose (µg/plate)	Strain(s)
N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	2	WP2uvraA
	3	TA100
	5	TA1535
2-nitrofluorene (2-NF)	0.2	TA98
9-aminoacridine hydrochloride	80	TA1537

Positive control, activation:

Positive control	Dose (µg/plate)	Strain
2-Aminoanthracene	0.5	TA98
	1	TA100
	2	TA1535, TA1537
	10	WP2uvrA

3. Activation: Rat liver S9 from Male Sprague-Dawley rats (5,6-benzoflavone induced)
   Source: Oriental Yeast Co., Ltd.
   Protein content: Not specified
   Characterisation: Not described

S9 mix composition:	
Na phosphate buffer (pH 7.4):	100 mM
Glucose-6-phosphate:	5 mM
NADP:	4 mM
NADPH:	4 mM
KCl:	33 mM
$MgCl_2$:	8 mM
S9:	10% (v/v)

4. Test organisms

Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* WP2*uvra* were checked for appropriate genetic markers and sensitivity after acquisition prior to use in the experiment.

5. Test concentrations for mutagenicity assay

Exp.1 and 2: (Pre-incubation)	Concentrations of 0, 313, 526, 1250, 2500 and 5000 µg IM-0/plate were evaluated in triplicate in the presence and absence of S9 activation.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

19 November – 6 December 1993

2. Main assay methods

This study consisted of 2 independent trials, with both Experiment 1 and Experiment 2 conducted using the pre-incubation method. In both experiments, test material/bacterial culture mixtures (0.1 mL each) and either phosphate buffer or S9 mixture (0.5 mL) were pre-incubated in water bath at 37°C for 20 minutes, then mixed with 2 mL soft agar and plated.

In both experiments, negative vehicle control, positive controls and five dose levels of the test substance were mixed with overnight cultures of *S.typhimurium* TA98, TA100, TA1535, TA1537 and *E.coli* WP2uvrA and plated on selective minimal agar in the presence and absence of 5,6-benzoflavone-induced rat liver S9 to evaluate the mutagenic potential of the test substance. All dose levels of test substance, negative vehicle control and positive controls were plated in triplicate. Plates were incubated for at 37°C until an appropriate time had passed to enable colony counting. No untreated control was used in this study.

Revertant colonies for a given tester strain and activation condition were counted by automated colony counter, by naked eye or by stereoscopic microscope. Growth inhibition and precipitate formation were observed visually with stereoscopic microscope.

3. Statistics

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated. Reporting of statistical evaluation was not provided.

4. Evaluation criteria

No acceptance criteria to demonstrate experimental validity were described.

Result interpretation criteria were described as follows:
- A result was considered positive where a 2-fold increase in the spontaneous mutation rate was observed compared with the solvent control, and where the result demonstrated a dose-response relationship and was reproducible.
- No specific negative result criteria were listed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Dimethyl sulfoxide (DMSO) was selected as the solvent of choice based on solubility data. In stability analyses, a 5% aqueous solution of IM-0 was described as stable for at least 4 hours.

B. MUTATION ASSAYS

In both experiments, no evidence of mutagenicity or bacteriotoxicity/growth inhibition were noted. In Experiment 1 (Table 1), test material precipitation was observed at 5000 µg/plate with and without S9 activation; however, this effect was not observed in Experiment 2 (Table 2).

Controls: The positive and vehicle controls fulfilled the requirements for a valid test.

Table 1
Summary of average revertants/plate after treatment with IM-0 without activation

Compound	Conc. µg/plate	TA98		TA100		TA1535		TA1537		WP2*uvrA*	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
IM-0	0	29 ± 8	28 ± 12	135 ± 5	134 ± 7	9 ± 4	10 ± 3	7 ± 5	6 ± 1	30 ± 4	17 ± 4
	313	21 ± 3	23 ± 4	124 ± 9	142 ± 5	15 ± 6	6 ± 2	5 ± 3	5 ± 2	25 ± 7	14 ± 2
	625	34 ± 5	24 ± 4	139 ± 9	128 ± 3	14 ± 3	6 ± 2	6 ± 2	4 ± 1	28 ± 3	18 ± 2
	125	31 ± 6	22 ± 2	145 ± 10	131 ± 4	13 ± 5	8 ± 4	3 ± 1	7 ± 3	28 ± 7	19 ± 3
	2500	30 ± 5	23 ± 4	140 ± 6	135 ± 7	9 ± 3	9 ± 3	9 ± 4	7 ± 2	26 ± 8	14 ± 3
	5000	26 ± 8	19 ± 5	141 ± 4	137 ± 11	10 ± 3	11 ± 2	6 ± 1	5 ± 2	30 ± 7	18 ± 5
ENNG	2/3/5	--	--	549 ± 34	517 ± 9	463 ± 19	489 ± 65	--	--	380 ± 32	301 ± 11
2-NF	0.2	89 ± 4	87 ± 3	--	--	--	--	--	--	--	--
9-AA	80	--	--	--	--	--	--	528 ± 17	615 ± 8	--	--

ENNG: N-ethyl-N'-nitro-N-nitrosoguanidine. 2-NF: 2-nitrofluorene; 9-AA: 9-aminoacridine hydrochloride

Data were derived from Tables 1-1 and 2-1, pp 19 and 21.

Table 2
Summary of average revertants/plate after treatment with IM-0 with activation

Compound	Conc. µg/plate	TA98		TA100		TA1535		TA1537		WP2*uvrA*	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
IM-0	0	29 ± 6	46 ± 3	132 ± 10	125 ± 6	10 ± 3	10 1	14 ± 5	13 ± 4	32 ± 4	26 ± 1
	313	37 ± 7	38 ± 7	140 ± 9	133 ± 11	8 ± 5	10 ± 3	7 ± 4	16 ± 4	40 ± 6	18 ± 3
	625	36 ± 6	44 ± 4	117 ± 22	110 ± 24	9 ± 2	8 ± 4	15 ± 4	21 ± 2	34 ± 8	23 ± 3
	125	43 ± 3	44 ± 2	135 ± 11	115 ± 10	14 ± 2	14 ± 7	16 ± 2	15 ± 4	32 ± 14	21 ± 1
	2500	33 ± 6	45 ± 3	146 ± 2	129 ± 14	15 ± 2	6 ± 1	17 ± 5	11 ± 3	28 ± 3	18 ± 3
	5000	40 ± 9	46 ± 4	149 ± 3	144 ± 4	13 ± 4	7 ± 2	13 ± 1	18 ± 8	29 ± 7	14 ± 5
2AA	Various*	174 ± 9	113 ± 9	560 ± 19	563 ± 2	197 ±16	203 ± 24	158 ± 8	144 ± 18	694 ± 10	574 ± 28

2AA= 2-aminoanthracene. See Section I.A.2 above for strain-specific concentrations used.

Data were derived from Tables 1–2 and 2–2, pp 20 and 22.

Primary review: Australia
Secondary review: US EPA &PMRA

B.6.8.3/09 *In vitro* genotoxicity—Bacterial Assay for Gene Mutation

Report:	Mochizuki, N. and Kanaguchi, Y. (1997b). IC-0: Reverse Mutation Study on Bacteria. Nippon Soda Co., Ltd, Toxicology Laboratory, Odawara Research Center, Kanagawa, Japan. Study ID C007440. Lab Project ID: G-942. Ref: M-195932-01-2. MRID 44988502. Unpublished.
Dates of work:	19 October – 15 November 1993; Final report 6 June 1994. 1st amendment 30 September 1997.
Guidelines:	EPA FIFRA Guideline 84-2 (1984), OECD No. 471 (1983), JMAFF 59 Nohsan No. 4200 (1985) and JMITI Guideline for reverse mutagenicity study in bacteria (1986).
GLP/QA:	Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary:

In independent trials of a reverse gene mutation assay, 6-chloronicotinic acid (IC-0) (99.14% Lot No. NK-3120) was evaluated for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2*uvrA* with and without an exogenous metabolic activation system (5,6-benzoflavone-induced rat liver S9) in two phases using the pre-incubation method (Experiment 1 and 2). The dose levels tested were 0, 313, 625, 1250, 2500 and 5000 µg/plate. The test substance was administered to the test system as a solution in dimethyl sulfoxide (DMSO).

In both experiments, growth inhibition of *Salmonella* bacterial strains and test material precipitation was observed across all strains at the limit dose of 5000 µg/plate with S9 activation. Test material precipitation was also observed in *E. coli* test strain at 5000 µg/plate with S9 activation.

Separately, across both experiments, growth inhibition of TA 98 bacteria was observed at 5000 µg/plate without S9 activation in the absence of test material precipitation.

The positive and vehicle controls fulfilled the requirements for a valid test. In the mutagenicity assay no positive mutagenic response occurred.

Under the conditions of this study, IC-0 was tested up to the limit dose and was negative for mutagenic activity in non-activated and S9-activated test systems.

The study is classified as **reliable (acceptable/guideline)** and satisfies the guideline requirements (OECD 471 and equivalents) for *in vitro* mutagenicity (bacterial reverse gene mutation) data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: IC-0 (synonym: 6-chloronicotinic acid)
   Lot/Batch #: NR.5
   Purity: 99.4%
   Description: Crystal
   CAS # 21543-49-7
   Stability of test compound: Stability analysis indicated that the test material was soluble (≥ 50 mg/mL in DMSO) and stable for 4 hours at 24°C as a 5 % aqueous solution in DMSO.
2. Control materials
   Solvent control: Dimethyl sulfoxide (DMSO) at 100 µL/plate for all materials

Positive control, non-activation:

Positive control	Dose (µg/plate)	Strain(s)
N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	2	WP2uvraA
	3	TA100
	5	TA1535
2-nitrofluorene (2-NF)	0.2	TA98
9-aminoacridine hydrochloride	80	TA1537

Positive control, activation:

Positive control	Dose (µg/plate)	Strain
2-Aminoanthracene	0.5	TA98
	1	TA100
	2	TA1535, TA1537
	10	WP2uvrA

3. Activation: Rat liver S9 from Male Sprague-Dawley rats (5,6-benzoflavone induced)
   Source: Oriental Yeast Co., Ltd.
   Protein content: Not specified
   Characterisation: Not described

S9 mix composition:	
Na phosphate buffer (pH 7.4):	100 mM
Glucose-6-phosphate:	5 mM
NADP:	4 mM
NADPH:	4 mM
KCl:	33 mM
$MgCl_2$:	8 mM
S9:	10% (v/v)

4. Test organisms

Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* WP2*uvra* were checked for appropriate genetic markers and sensitivity after acquisition prior to use in the experiment.

5. Test concentrations for mutagenicity assay

Exp.1 and 2: (Pre-incubation)	Concentrations of 0, 313, 526, 1250, 2500 and 5000 µg IC-0/plate were evaluated in triplicate in the presence and absence of S9 activation.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

19 October – 15 November 1993

2. Main assay methods

This study consisted of 2 independent trials, with both Experiment 1 and Experiment 2 conducted using the pre-incubation method. In both experiments, test material/bacterial culture mixtures (0.1 mL each) and either phosphate buffer or S9 mixture (0.5 mL) were pre-incubated in water bath at 37°C for 20 minutes, then mixed with 2 mL soft agar and plated.

In both experiments, negative vehicle control, positive controls and five dose levels of the test substance were mixed with overnight cultures of *S.typhimurium* TA98, TA100, TA1535, TA1537 and *E.coli* WP2uvrA and plated on selective minimal agar in the presence and absence of 5,6-benzoflavone-induced rat liver S9 to evaluate the mutagenic potential of the test substance. All dose levels of test substance, negative vehicle control and positive controls were plated in triplicate. Plates were incubated for at 37°C until an appropriate time had passed to enable colony counting. No untreated control was used in this study.

Revertant colonies for a given tester strain and activation condition were counted by automated colony counter, by naked eye or by stereoscopic microscope. Growth inhibition and precipitate formation were observed visually with stereoscopic microscope.

3. Statistics

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated. Reporting of statistical evaluation was not provided.

4. Evaluation criteria

No acceptance criteria to demonstrate experimental validity were described.

Result interpretation criteria were described as follows:
- A result was considered positive where a 2-fold increase in the spontaneous mutation rate was observed compared with the solvent control, and where the result demonstrated a dose-response relationship and was reproducible.
- No specific negative result criteria were listed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Dimethyl sulfoxide (DMSO) was selected as the solvent of choice based on solubility data. In stability analyses, a 5% aqueous solution of IC-0 was described as stable for at least 4 hours.

B. MUTATION ASSAYS

In both experiments, no evidence of mutagenicity was observed (i.e. no increased frequency of revertant colonies with test material treatment).

In both experiments, growth inhibition of *Salmonella* bacterial strains and test material precipitation was observed across all strains at 5000 μg/plate with S9 activation. Test material precipitation was also observed in *E. coli* test strain at 5000 μg/plate with S9 activation (Tables 1–2).

Separately, across both experiments, growth inhibition of TA98 bacteria was observed at 5000 μg/plate without S9 activation without the presence of test material precipitation.

Controls: The positive and vehicle controls fulfilled the requirements for a valid test.

Table 1
Summary of average revertants/plate after treatment with IC-0 without activation

Compound	Conc. µg/plate	TA98		TA100		TA1535		TA1537		WP2*uvrA*	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
IC-0	0	24 ± 4	24 ± 4	127 ± 3	127 ± 3	10 ± 7	12 ± 3	11 ± 3	14 ± 3	19 ± 3	24 ± 3
	313	26 ± 2	27 ± 4	116 ± 5	125 ± 16	8 ± 3	9 ± 3	14 ± 3	11 ± 2	17 ± 4	22 ± 7
	625	26 ± 4	35 ± 2	109 ± 9	132 ± 5	6 ± 1	11 ± 2	14 ± 5	10 ± 4	22 ± 5	27 ± 6
	125	25 ± 2	25 ± 1	135 ± 5	138 ± 5	8 ± 1	9 ± 6	19 ± 5	13 ± 5	20 ± 6	26 ± 6
	2500	30 ± 3	31 ± 9	122 ± 11	134 ± 4	8 ± 3	15 ± 4	12 ± 4	14 ± 5	24 ± 3	20 ± 4
	5000	10 ± 5	18 ± 7	134 ± 9	134 ± 9	4 ± 2	9 ± 3	10 ± 4	12 ± 2	18 ± 3	23 ± 3
ENNG	2/3/5	--	91 ± 9	557 ± 25	657 ± 25	495 ± 66	516 ± 25	--	--	445 ± 38	436 ± 9
2-NF	0.2	88 ± 5		--	--	--	--	--	--	--	--
9-AA	80	--	--	--	--	--	--	548 ± 27	574 ± 40	--	--

ENNG: N-ethyl-N'-nitro-N-nitrosoguanidine. 2-NF: 2-nitrofluorene; 9-AA: 9-aminoacridine hydrochloride

Data were derived from Tables 1-1 and 2-1, pp 19 and 21.

Table 2
Summary of average revertants/plate after treatment with IC-0 with activation

Compound	Conc. µg/plate	TA98		TA100		TA1535		TA1537		WP2*uvrA*	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
IC-0	0	46 ± 7	35 ± 3	127 ± 5	120 ± 6	14 ± 1	15 ± 2	20 ± 2	12 ± 5	32 ± 12	27 ± 5
	313	47 ± 2	43 ± 9	121 ± 7	124 ± 16	10 ± 4	17 ± 5	15 ± 3	19 ± 2	36 ± 4	24 ± 2
	625	42 ± 2	45 ± 17	113 ± 10	107 ± 6	14 ± 2	16 ± 5	14 ± 4	14 ± 4	28 ± 6	21 ± 4
	125	38 ± 2	39 ± 9	129 ± 14	130 ± 10	11 ± 6	11 ± 3	13 ± 2	14 ± 4	31 ± 11	28 ± 6
	2500	34 ± 7	39 ± 3	133 ± 9	114 ± 4	22 ± 6	15 ± 3	20 ± 2	17 ± 3	22 ± 5	23 ± 5
	5000	13 ± 6	18 ± 1	90 ± 22	113 ± 11	9 ± 2	22 ± 2	10 ± 4	18 ± 4	28 ± 10	28 ± 5
2AA	Various*	154 ± 4	125 ± 15	592 ± 23	652 ± 20	184 ± 9	201 ± 18	164 ± 8	148 ± 9	663 ± 14	682 ± 15

2AA= 2-aminoanthracene. See Section I.A.2 above for strain-specific concentrations used.

Data were derived from Tables 1–2 and 2–2, pp 20 and 22.

III. EVALUATION, SUMMARY and CONCLUSIONS by REGULATORY AUTHORITY

A. NAME OF AUTHORITY: Office of Chemical Safety/Department of Health and Ageing (Australia).

B. REVIEWER'S COMMENTS:

RELIABILITY RATING: Reliable

This study is compliant with OECD 471(1983) and other internationally-recognised equivalent test guidelines.

C. CONCLUSIONS: IC-0 was negative for mutagenic activity up to the limit dose of 5000 µg/plate in all strains in both trials in the presence and absence of S9-activation in the in vitro bacterial gene mutation assay. There were no treatment-related increases in the mean number of revertants/plate in any strain (±S9). The positive controls induced marked increases in revertant colonies compared to controls in all strains in the presence and absence of S9 activation.

Accordingly, IC-0 is negative in this test system for mutagenicity.