Document Title

Summary of the Toxicological and Metabolism studies for Isoflucypram

(Code: BCS-CN88460)

Data Requirement(s)

Regulation (EC) No 1107/2009 & Regulation (EU) No 283/2013

Document MCA

Section 5: Toxicological and Metabolism studies

According to the Guidance Document SANCO/10181/2013 for applicants on preparing dossiers for the approval of a chemical active substance

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Author(s)

[redacted]

[redacted]

Bayer AG

Crop Science Division

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Version history

Date [yyyy-mm-dd]	Data points containing amendments or additions[1] and brief description	Document identifier and version number
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2018-04-16	Dossier update - CA 5.1: Additional information added - CA 5.2: New references M-488523-01-1 and M-613506-02-1 added in overview table - CA 5.2.4: Additional information added - CA 5.2.5/02: New study M-488523-01-1 and text added - CA 5.2.7: Final Report M-613506-02-1and text added - CA 5.3: Additional information added - CA 5.4.2/01: Additional information added - CA 5.5/01: Final Report M-612739-02-1 added - CA 5.6.1/01: Final Report M-612750-02-1 and additional information added - CA 5.8.1/01: New Document M-612432-01-1 added - CA 5.8.1/02: Additional information added - CA 5.8.2/02: New study M-615229-01-1 added - CA 5.8.2/03: New study M-614504-01-1 added - CA 5.8.2/04: New study M-615222-01-1 added	M-613612-02-1

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

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

INTRODUCTION

Isoflucypram (CAS-No. 1255734-28-1) is a new fungicidal active substance developed by Bayer.

This document supports the application for regulatory approval of isoflucypram in Europe under Regulation (EC) No 1107/2009.

The document MCA Section 5 summarises all toxicological and metabolism data, risk assessments and classification proposal which are relevant for the approval of isoflucypram alongside the proposed intended uses, including the representative uses, under Regulation (EC) No 1107/2009 in accordance with the requirements laid down in the Commission Regulation (EU) No 283/2013 and under Classification Regulation (EC) No 1272/2008.

Isoflucypram is a novel broad spectrum fungicide of the chemical class of N-cyclopropyl-N-benzyl-pyrazole-carboxamides with an outstanding efficacy against the major economically important fungal diseases of cereal crops (wheat, triticale, rye, barley and oats) and excellent crop safety.

Since isoflucypram is an SDH inhibitor and thus assigned to the FRAC resistance Group 7 the application scope of isoflucypram-containing products on cereals with only one foliar spray at a maximum of 75 g a.s./ha supports an effective anti-resistance management strategy.

Tailor-made and broad spectrum isoflucypram combinations show highly beneficial properties in terms of plant physiology beside the long-lasting and certain curative efficacy to control fungal diseases and to maximize the full yield potential of the cereal crops.

Details of the literature search undertaken are summarized in MCA Section 9. For isoflucypram and its metabolites, no publication and relevant scientifically peer-reviewed open literature reference has been identified which would indicate that a side-effect on human health, the environment and non-target species may exist, which would then need to be considered in the risk assessment of this new active substance dossier.

Throughout the development of isoflucypram the following synonyms may have been used and also referred to in individual study reports: Bayer Code: BCS-CN88460, BCS-CN88460-a.s., ´460 and the Bayer-internal short Code: ISY. All chemical substances described by either of these codes refer to the same chemical name and structural formula.

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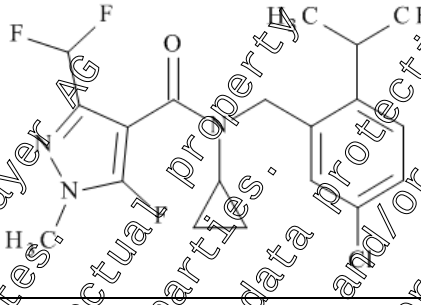
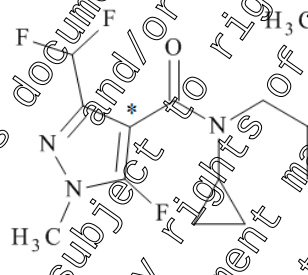
CA 5.1 Studies on absorption, distribution, metabolism and excretion in mammals

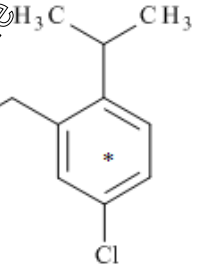
The absorption, distribution, excretion and metabolism of isoflucypram (BCS-CN88460) in rats were investigated. The chemical structure and nomenclature are provided below.

Chemical structure	
Common name	Isoflucypram
Company experimental name	BCS-CN88460
IUPAC name	N-(5-chloro-2-isopropylbenzyl)-N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazole-4-carboxamide
Molecular formula	$C_{19}H_{21}ClF_3N_3O$
Molecular weight	399.8 g/mol

As isoflucypram contains separate ring systems, two different radiolabels were used in the rat studies. These label positions are shown below:



[pyrazole-4-^{14}C]



[phenyl-UL-^{14}C]

Five different studies were performed and are summarised in chapter CA 5.1.1:
- two ADME studies with the two different labels
- two quantitative whole body autoradiography studies with the two different labels, one of them additionally encompassing a pilot metabolism study
- one metabolism study with a metabolite of BCS-CN88460, namely [pyrazolyl-4-^{14}C]BCS-CY26497 (report name: BCS-CN88460-carboxylic acid)

Numerous metabolites were identified in these studies. The chemical structures and report names used in the reports and summaries are given in the List of Metabolites which is a separate document of this dossier (Document N3). An evaluation of metabolites conclusively identified in plants and livestock including toxicity assessment, metabolite occurrence and coverage in rat ADME studies is provided in a separate document M-612432-01-1 referenced in this MCA Summary Section 5 under Point 5.8.1 and metabolite occurrences are as well summarized in Document N1.

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An *in vitro* comparative metabolism study has been conducted with [pyrazole-4-^{14}C]BCS-CN88460 using rat, mice, human, dog and rabbit liver microsomes. This study was conducted to demonstrate that the biotransformation of isoflucypram is comparable in human, mice and rat. The study is summarised in chapter CA 5.1.2.

The results from the performed five different rat studies are in good agreement to each other and significant differences in the biokinetic and metabolism behaviour of isoflucypram were not observed. The absorption was fast (about 1 hour) for all low dose tests (2 mg/kg bw). In high dose tests with 200 mg/kg bw, the mean maximum plasma concentration was measured at 2 h after dosing for male rats and at 4 h after dosing for female rats in the course of time. The excretion of radioactivity was completed at latest 72 hours after administration. Fecal excretion was predominantly higher than the renal excretion and ranged between 78 and 96% of the administered dose, except for bile-duct cannulated rats (21% for males and 16% for females). Approximately 74% of the mean dose recovered was detected in bile samples of male bile-duct cannulated rats and approximately 82% in bile samples of females, respectively. For all tests the mean urinary excretion rate ranged between 6 and 14% of the administered dose, except for bile-duct cannulated female rats with a urinary excretion rate of about 2% of the administered dose. Based on the recovered radioactivity detected in bile, urine and bodies without GIT, the absorption rates were calculated and amounted to 80% for males and 84% for females.

The results of the quantitative whole-body autoradiography studies demonstrated that the radioactivity was distributed among all tissues. The absorbed test compound related radioactivity was distributed throughout the animal bodies immediately after dosing with a clear preference to the liver and kidney that are the responsible organs for metabolism and excretion, respectively. No relevant sex related differences concerning the maximum equivalent concentrations in blood, organs, and tissues was observed in male or female rats. Absorbed radioactivity was quickly and efficiently eliminated from the bodies of the rats of both genders within 72 h after administration and long term retention of residues in any of the organs and tissues can therefore be excluded.

The unchanged parent compound was detected between 0.73% and 39.07% of the administered dose and was only present in faeces, and in small amounts in liver and kidney. Qualitative and quantitative differences were observed in the metabolic profiles of male and female rats in the low and high dose tests. A high number of various metabolites was characterised and identified in samples of male and female rats. No relevant qualitative or quantitative differences in the metabolic profile of pre-treated male rats were observed in comparison to single dosing. The metabolite pattern was similar in all studies for common metabolites and in good agreement for the label-specific metabolites.

The most important metabolic reactions of [pyrazole-4-^{14}C]BCS-CN88460 were the demethylation of the pyrazole moiety and the hydroxylation leading to mono-, di or tri-hydroxy compounds followed by conjugation with amino acids, glucuronic acid and sulphuric acid. Another important metabolic reaction was the carboxylation of the 1-propanol group, leading to a carboxylic acid or with a hydroxy group in position 2 of the propyl group to a lactic acid group. Conjugation of hydroxy compounds with glucuronic acid was also prominent. Conjugation with sulphuric acid was observed. Further observed was the conjugation with glutathione after defluorination followed by degradation of the glutathione conjugate to glycine-cysteine or cysteine conjugates. Conjugation of the BCS-CN88460-pyrazole-carboxylic acid compound with alanine and conjugation of the BCS-CN88460-desmethyl with sulphuric acid were detected. Cleavage of the pyrazole moiety was leading to a number of benzyl alcohol compounds and BCS-CN88460-phenyl-formyl-olefine. Additional minor reactions were the oxidation of the 1-propanol group, cleavage of the phenyl moiety, cleavage of the phenyl moiety in combination with cleavage of the cyclopropyl ring, dehydration after hydroxylation of the propyl group and oxidation in the pyrazole ring.

Major metabolites in male and female rats were BCS-CN88460-carboxylic acid, BCS-CN88460-desmethyl-carboxylic acid, BCS-CN88460-desmethyl-hydroxyphenyl-1,2-propandiol, BCSCN88460-

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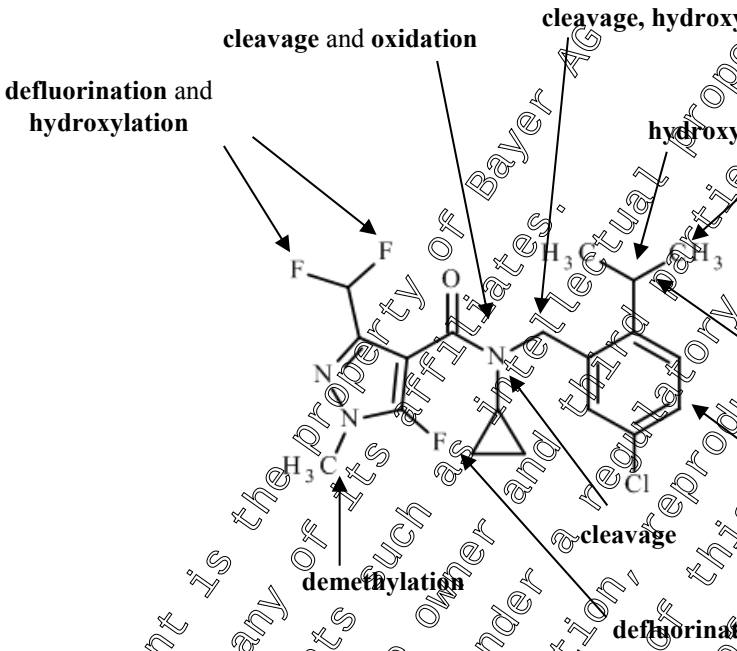
desmethyl-OH-GlucA (isomer 2), BCS-CN88460-desmethyl, BCS-CN88460-desmethyl-hydroxy-methyl-carboxylic acid, BCS-CN88460-desmethyl-propanol, BCS-CN88460-desmethyl-GlucA (isomer 1 and 2), BCS-CN88460-propanol, BCS-CN88460-propanol-GlucA (isomer 1), BCS-CN88460-desmethyl-diOH (group of isomers) and BCS-CN88460-desmethyl-diOH-GlucA (isomer 1).

The following figure shows schematically the positions in the molecule, which are mainly involved in the metabolic reactions.



Note:
Non-specific hydroxylation or conjugation with glucuronic acid, amino acids, or sulphuric acid was not depicted, as the exact position could not be located by structure elucidation.

BCS-CY26497 (report name: BCS-CN88460-carboxylic acid) one of the major metabolites of isoflucypram, labelled with ^{14}C in the pyrazolyl-4 moiety of the molecule, was absorbed very fast from the gastrointestinal tract after single oral administration. The highest plasma level was measured at 0.25 hours. Excretion of radioactivity was all most via faeces and only a minor part with urine. Excretion was nearly completed within 24 hours.
BCS-CY26497 was the main compound in the extract of faeces. The major amount of follow-up metabolites from BCS-CY26497 was excreted predominantly via the faecal route, but the major number of metabolites was detected in the urine, representing very low amounts.
Demethylation of the pyrazole moiety and hydroxylation in the propionic acid group were the most prominent metabolic reactions. Cleavage of the phenyl moiety and the cyclopropyl ring were observed as prominent reactions. Further conjugation with glucuronic acid after hydroxylation or via nitrogen and conjugation with alanine was found.

[Pyrazole-4-^{14}C]BCS-CN88460 was incubated with liver microsomes from humans, rats, mice, dogs and rabbits. The biotransformation rate of radiolabelled isoflucypram and the metabolic pattern was found to be moderately different in the various microsomal incubations. The enzymatic activity of each of the liver microsomes was demonstrated by a significant metabolic conversion of the positive control substance ^{14}C-testosterone. Beside BCS-CN88460 up to twenty-one metabolites were detected in the liver microsomes incubates after biotransformation of [pyrazole-4-^{14}C]BCS-CN88460. The metabolic pattern in all liver microsomes was qualitatively comparable. All metabolites formed by

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microsomes from humans were also detectable in microsomes from rat and no human unique metabolites were detected. The metabolic profiles of the *in-vitro* rat liver microsomes were in good accordance to the metabolite profiles of faeces pool and bile pool samples of male rats after single low dose administration of 2 mg/kg [pyrazole-4-^{14}C]BCS-CN88460.

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

The absorption, distribution, excretion and metabolism of isoflucypram (BCS-CN88460) and BCS-CY26497 (report name: BCS-CN88460-carboxylic acid), one of the major metabolites of isoflucypram, in rats were investigated in five different studies, for which details are presented in the following table.

Type of study	Dose level	Animal species, strain; sex	Substance	Reference
ADME study	2.0 mg/kg bw (single low dose and repeated low dose) or 200 mg/kg bw (single high dose)	Male and female Wistar (Hsd/Cpb: WU) rats	pyrazole-labelled Isoflucypram	M-602452-02-1
ADME study	2.0 mg/kg bw (single dose)	Male and female Wistar (Hsd/Cpb: WU) rats	phenyl-labelled Isoflucypram	M-602883-02-1
Whole-body autoradio-graphic distribution	5.0 mg/kg bw (single dose)	Male and female Wistar (Hsd/Cpb: WU) rats	pyrazole-labelled Isoflucypram	M-602456-01-1
Whole-body autoradio-graphic distribution	5.0 mg/kg bw (single dose)	Male and female Wistar (Hsd/Cpb: WU) rats	phenyl-labelled Isoflucypram	M-590199-02-1
Metabolism study	5.0 mg/kg bw (single dose)	Male Wistar (Hsd/Cpb: WU) rats	[pyrazolyl-4-^{14}C] BCS-CY26497	M-604147-02-1

The first study report ([redacted], R.; [redacted], N.; 2017; M-602452-02-1) describes the absorption, distribution, metabolism, and excretion of the [pyrazole-4-^{14}C] labelled test item in male and female rats which were dosed with a single low dose, with a single high dose experiments, with a single low dose after bile-duct cannulation, and a single low dose after 14 days pre-treatment with non-radiolabelled test item (males only). The toxicokinetic behavior of the total radioactivity was investigated by plasma curve analysis.

The second report ([redacted], R.; [redacted], N.; 2017; M-602883-02-1) describes the absorption, distribution, metabolism, and excretion of the [phenyl-UL-^{14}C] labelled test item in male and female, rats, which were dosed with a single low dose. The toxicokinetic behavior of the total radioactivity was investigated by plasma curve analysis.

The third study report ([redacted], R.; [redacted], N.; 2017; M-602456-01-1) describes the distribution of the total radioactivity of [pyrazole-4-^{14}C] labelled isoflucypram in male and female rats by quantitative whole body autoradiography, the determination of the exhaled $^{14}CO_2$ and the pilot investigations of the metabolism after a single low dose administration to male and female rats. Parent compound and metabolites were identified in samples from urine and plasma and from extracts of liver, kidney and faeces.

The fourth study report ([redacted]; [redacted]; 2017; M-590199-02-1) describes the distribution of the total radioactivity of the [phenyl-UL-^{14}C] labelled test item in male and female rats by quantitative whole body autoradiography after a single low dose of 5 mg/kg bw. The excretion of

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radioactivity was investigated in urine and faeces and the radioactivity concentration was determined in the organs and tissues at sacrifice. The toxicokinetic behavior of the total radioactivity was investigated by plasma curve analysis.

The fifth study report ([redacted], R., [redacted], N.; 2017; M-604147-02-1) describe the absorption, distribution, metabolism and excretion of [pyrazolyl-4-^{14}C] labelled BCS-CY26497 (report name: BCS-CN88460-carboxylic acid), one of the major metabolites of isoflucypram, in male and female rats after a single oral low dose of 5 mg/kg bw. The excretion of radioactivity was investigated in urine and faeces and the radioactivity concentration was determined in plasma and blood cells at sacrifice. Metabolites were identified in urine and faeces. The toxicokinetic behavior of the total radioactivity was investigated by plasma curve analysis.

Report:	KCA 5.1.1/01; [redacted], R.; [redacted], N.; 2017; M-602452-02-1
Title:	Amendment no 1 to final report - [Pyrazole-4-14C]BCS-CN88460 - Absorption, distribution, excretion and metabolism in the rat
Report No.:	EnSa-16-1015
Document No.:	M-602452-02-1
Guideline(s):	Regulation (EC) No 1107/2009 of the European Parliament and of the Council amended by Commission Regulation (EU) No 283/2013 US EPA OCSPP 870.7485 Japanese MAFF Test Guideline 12 Nousan 8147
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

The toxicokinetic behaviour (absorption, distribution, and excretion) and metabolism of BCS-CN88460, labelled with ^{14}C in pyrazole-4 moiety of the molecule, was investigated in male and female Wistar rats.

Seven experiments were conducted in this study encompassing low dose (2 mg/kg bw) and high dose (200 mg/kg bw) and low dose repeated treatment. The rats received the radioactive test compound by oral gavage as a suspension in water and Tragacanth at pH 4. They were sacrificed three days post dosing and in case of bile-duct cannulation two days post dosing. The total radioactivity included the radioactivity related to the test compound and the metabolites and was determined in plasma, urine, bile, faeces, organs and tissues samples at sacrifice. Metabolites were investigated in urine, bile and extracts of faeces.

Recovery

Between 100.3% and 104.5% of the administered radioactivity were recovered by measurement of the total radioactivity in plasma samples, urine and faeces, as well as in bile, organs and tissues and plasma samples at sacrifice.

Absorption

The absorption of [pyrazole-4-^{14}C]BCS-CN88460 started immediately after administration and the maximum plasma concentration (C_{max}) was reached within 1 h (t_{max}) post administration for the low dose tests (2 mg/kg bw) for both, male and female rats. In the high dose tests with 200 mg/kg bw, the mean maximum plasma concentration was measured at 2 h after dosing for male rats and at 4 h after dosing for female rats. The time course of the mean plasma levels was comparable in male and female rats in all tests.

Low dose tests with bile-duct cannulated male and female rats showed that about 74% of the dose recovered was detected in the bile of male animals, and 82% in the bile of females. Absorption rates

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were calculated by summation of the recovered radioactivity in urine, bile, and body without GIT and amounted to 79.9% for male rats and 84.1% for female rats.

Distribution and plasma kinetics

From peak levels, the time course of radioactivity in plasma showed a decline and efficient elimination of the test substance and its metabolites from the body at the latest within 72 h post administration. The plasma concentration in the low dose tests as well as the high dose tests were calculated and showed a fast elimination phase after reaching the plasma peak followed by a slower elimination phase after approximately 24 h. There were no sex specific differences in the calculated AUC_0 - t values for low or high dose male and female rats. However, rats dosed with 2 mg/kg bw showed an approximately 1.75-times higher exposure compared to rats dosed with 200 mg/kg bw due to the lower absorption at higher dose levels.

Excretion

Generally, in the low and high dose tests the excretion was almost completed 72 h after administration. At this time more than 98% of the recovered dose had been excreted via urine and faeces. In all low and all high dose tests the main portion of radioactivity (~80%) was excreted latest after 24 h.

In all tests the excretion was predominantly faecal. Faecal excretion of individual rats ranged from about 83% to 96% of the recovered radioactivity, with exception of the tests with bile-duct cannulated rats. In bile-duct cannulated rats, approximately 20% of the recovered dose (mean value) was detected in faeces of male rats and approximately 15% in faeces of females. Approximately 74% of the mean dose recovered was detected in bile samples of male bile-duct cannulated rats and approximately 82% in bile samples of females, respectively.

For single low dose tests the mean urinary excretion rate was 13.6% of the dose recovered for male rats and 12.0% for female rats. For high dose tests mean values of 7.1% of the dose were recovered in the urine of male rats and 8.4% of the dose in the urine of female rats. The lower urinary excretion rates at the high dose tests give a hint, that there is a lower absorption of BCS-CN88460 at higher dose rates.

Residues in organs and tissues at sacrifice

Generally, there were moderate radioactive residues in organs and tissues of all tests. In the low dose tests female rats showed lower organ concentrations compared to male rats. At sacrifice low levels of radioactivity (up to 0.365% of the dose) were found in the bodies excluding GIT. Low amounts of radioactivity were detected in the GITs (up to 0.044%), excluding GITs from bile-duct cannulated rats. Compared to low dose tests, lower organ concentrations were observed in repeated and in high dose tests.

For all tests, the highest concentration of radioactivity in organs and tissues was detected in the liver and blood cells.

Metabolism

A high number of metabolites was identified and characterised in the excreta and bile of male and female rats, suggestive of an intensive metabolism of BCS-CN88460 in the rat. The majority of metabolites (between 85.61% and 100.79%) was identified (see table below). The other metabolites were characterised by their extraction and chromatographic behaviour. Probably more isomeric forms of identified metabolites may have been formed as indicated by broad non resolved zones in the chromatograms.

Qualitative and quantitative differences were observed in the metabolic profiles of male and female rats in the low and high dose tests. A high number of various metabolites was characterised and identified in samples of male and female rats. No relevant qualitative or quantitative differences in the metabolic profile of pre-treated male rats were observed in comparison to single dosing.

The unchanged parent compound was detected between 0.73% and 59.07% of dose and was only present in faeces. The most important metabolic reactions of [pyrazole-4-^{14}C]BCS-CN88460 were the

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demethylation of the pyrazole moiety and the hydroxylation leading to mono-, di or-tri-hydroxy compounds followed by conjugation with amino acids, glucuronic acid, and sulphuric acid. Another important metabolic reaction was the carboxylation of the 1-propanol group, leading to a carboxylic acid or with a hydroxy group in position 2 of the propyl group to a lactic acid group.

Major metabolites in male and female rats were BCS-CN88460-carboxylic acid, BCS-CN88460-desmethyl-carboxylic acid. BCS-CN88460-desmethyl-hydroxyphenyl-1,2-propandiol and BCSCN88460-desmethyl-OH-GlucA (isomer 2), BCS-CN88460-desmethyl, BCS-CN88460-desmethyl-hydroxymethyl-carboxylic acid, BCS-CN88460-desmethyl-propanol, BCS-CN88460-desmethyl-GlucA (isomer 1 and 2), BCS-CN88460-propanol-GlucA (isomer 1) and BCS-CN88460-desmethyl-diOH-GlucA (isomer 1) accounting for up to 18.84% of the administered dose.

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Table 5.1.1- 1: Test 1, 2, 3, 4, 5, 6 and 7: Balance of BCS-CN88460 and metabolites excreted with urine, bile and faeces after oral treatment to male and female rats

Report name BCS-CN88460-	Test 1 (male, 2 mg/kg bw)	Test 2 (female, 2 mg/kg bw)	Test 3 (male, 200 mg/kg bw)	Test 4 (female, 200 mg/kg bw)	Test 5 (male, pre-treatment, 2 mg/kg bw)	Test 6 (male, bile-duct can., 2 mg/kg bw)	Test 7 (female, bile-duct can., 2 mg/kg bw)
	% of dose administered						
pyrazole-amide	0.86	0.08	0.31	---	0.50	---	---
cyclopropyl-pyrazole-carboxamide-OH-GlucA	0.72	0.08	0.18	0.07	0.43	0.28	---
pyrazole-carboxylic acid	1.59	0.18	0.16	0.10	1.02	0.74	---
cyclopropyl-pyrazole-carboxamide-GlucA (isomers)	2.85	0.80	0.89	0.53	2.36	3.37	0.25
desfluoro-N-methyl-pyrazole-carboxylic acid	0.50	---	0.19	---	0.41	---	0.10
pyrazole-carboxylic acid-Ala	4.92	1.69	3.10	1.18	4.77	1.70	0.16
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys and desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys-Gly	---	---	---	---	---	1.57	0.24
N-methyl-pyrazole-carboxylic acid	0.67	0.37	0.30	0.37	0.50	0.26	0.13
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH	---	---	---	---	---	0.94	0.04
cyclopropyl-pyrazole-carboxamide	0.76	0.60	1.63	0.40	1.67	1.00	0.13
cyclopropyl-oxy-pyrazole-carboxamide	0.31	---	---	---	0.27	0.10	---
desmethyl-triOH-GlucA	0.98	---	---	---	0.54	2.80	---
desmethyl-diOH-GlucA (isomer 1)	1.12	---	---	---	---	5.56	---
desmethyl-diOH-GlucA (isomer 2 and isomer 3)	---	---	---	---	---	0.85	---
desmethyl-hydroxymethyl-diOH	2.86	---	---	---	1.55	0.36	0.66
desmethyl-diOH-GlucA (isomer 3)	0.52	---	---	---	0.31	1.52	1.42
desmethyl-hydroxyphenyl-1,2-propandiol	8.11	---	0.76	---	6.07	---	---
desmethyl-diOH-GlucA (isomer 4)	---	---	---	---	---	2.17	2.71
desmethyl-diOH-GlucA (isomer 5)	---	---	---	---	---	1.45	2.65
desmethyl-OH-GlucA (isomer 1)	---	---	---	---	---	1.90	0.20
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	1.81	1.16	---	---	1.56	1.64	2.13
desmethyl-OH-GlucA (isomer 2)	10.24	0.23	2.22	0.76	10.47	2.09	3.66
desmethyl-hydroxyphenyl-2-propanol	4.32	6.74	3.74	2.03	4.18	1.44	1.69
desmethyl-lactic acid	1.98	---	---	---	1.52	3.50	4.36
desmethyl-hydroxymethyl-carboxylic acid	3.01	11.38	2.31	4.12	6.15	0.20	0.21
desmethyl-diOH (isomer)	1.57	---	0.47	---	0.55	0.06	---

Table is continued on next page

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Table 5.1.1- 1 continued

Report name BCS-CN88460-	Test 1 (male, 2 mg/kg bw)	Test 2 (female, 2 mg/kg bw)	Test 3 (male, 200 mg/kg bw)	Test 4 (female, 200 mg/kg bw)	Test 5 (male, pre-treatment, 2 mg/kg bw)	Test 6 (male, bile-duct can., 2 mg/kg bw)	Test 7 (female, bile-duct can., 2 mg/kg bw)
	% of dose administered						
desmethyl-oxo-GlucA, carboxylic acid-GlucA and desmethyl-carboxylic acid-GlucA (isomer 2)	---	---	---	---	---	4.58	4.58
desmethyl-propanol-GlucA (isomer 1), olefine, oxo-GlucA, lactic acid and desmethyl-diOH-GlucA (isomer 6)	---	---	---	---	---	13.24	11.94
lactic acid	2.60	2.11	1.18	0.39	2.20	---	---
propanol-GlucA (isomer 1 and 2)	1.17	---	---	---	1.31	---	---
propanol-GlucA (isomer 1)	---	---	---	---	---	6.98	12.27
propanol-GlucA (isomer 2)	---	---	---	---	---	3.47	3.46
desmethyl-SA	0.28	---	---	---	---	0.19	0.08
desmethyl-carboxylic acid	12.44	18.84	10.23	7.76	13.76	1.51	2.94
desmethyl-propanol	1.60	8.68	0.22	3.34	4.43	0.59	1.23
carboxylic acid	12.61	13.77	5.80	6.62	14.34	2.68	1.54
propanol	0.95	4.09	0.81	2.79	2.44	0.20	0.05
desmethyl-GlucA (isomer 1)	---	---	---	---	---	3.86	14.09
desmethyl-GlucA (isomer 2)	---	---	---	---	---	1.64	7.26
2-propanol	0.70	0.64	0.83	0.59	1.19	0.07	0.04
desmethyl-propanol-GlucA (isomer 2)	---	---	---	---	---	0.77	4.55
desmethyl	1.44	11.43	2.74	5.31	2.98	0.38	0.46
parent compound	4.11	4.86	57.26	59.07	0.73	18.24	15.56
Total identified	**87.66**	**85.61**	**90.34**	**95.47**	**88.17**	**93.92**	**100.79**
Total characterised	**9.87**	**10.61**	**1.45**	**3.05**	**10.03**	**7.85**	**2.20**
Number of characterised metabolites	21	8	5	5	13	20	9
Maximum value of a characterised metabolite	2.02	3.52	0.59	1.32	1.95	1.46	0.63
Exhaustive extract of faeces	2.98	1.86	---	---	1.90	---	---
Solids of faeces (PES)	2.58	1.84	2.59	1.92	2.18	0.59	0.35
Urine not analysed	0.06	0.07	0.03	0.03	0.06	0.35	---
Bile not analysed	---	---	---	---	---	1.03	---
Faeces not analysed	0.20	0.14	0.14	0.07	0.30	0.43	---
Fractions not analysed	---	---	---	---	0.04	0.01	---
Total	**103.34**	**100.13**	**100.55**	**100.54**	**102.68**	**104.16**	**103.34**

can. = cannulated
--- = not detected

In general, the main metabolic routes observed for rats treated with [pyrazole-4-^{14}C]BCS-CN88460 and [phenyl-UL-^{14}C]BCS-CN88460 were identical. Cleavage of the phenyl moiety led to pyrazole label specific metabolites, which were not detected in the phenyl label study.

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The principal metabolic reactions of [pyrazole-4-^{14}C]BCS-CN88460 in the rat are listed below:

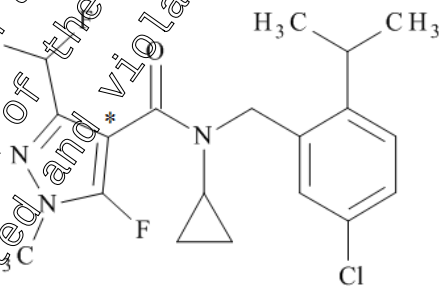
- demethylation of the pyrazole moiety
- hydroxylation in position 1 and position 2 of the propyl group, in the phenyl ring, and after defluorination of the difluoromethyl moiety was leading to mono-, di- or tri-hydroxy compounds. Hydroxylation in other positions was also detected, but was not exactly located by structure elucidation.
- further oxidation of the 1-propanol group was leading to a carboxylic acid group or in combination with a 2-propanol group to a lactic acid group
- cleavage of the phenyl moiety was leading to cyclopropyl-pyrazole-carboxamide compounds
- cleavage of the phenyl moiety in combination with cleavage of the cyclopropyl ring was leading to a pyrazole-amide compound followed by oxidation to carboxylic acid compounds
- conjugation with glucuronic acid after hydroxylation or via nitrogen was leading to several glucuronic acid-conjugates
- conjugation with glutathione after defluorination of the cyclopropyl-pyrazole-carboxamide moiety followed by degradation of the glutathione conjugate to glycine-cysteine or cysteine conjugates
- conjugation of the BCS-CN88460-pyrazole-carboxylic acid compound with alanine
- conjugation of the BCS-CN88460-desmethyl with sulphuric acid
- dehydration after hydroxylation of the propyl group was leading to BCS-CN88460-olefine
- oxidation in the pyrazole ring was leading to BCS-CN88460-cyclopropyl-oxy-pyrazole-carboxamide

Based on these results it is concluded that the biokinetic and metabolic behaviour of BCS-CN88460 in rats is sufficiently understood and a metabolic pathway is proposed.

I. MATERIALS AND METHODS

A. Material

1. Test material:

Test substance	
Chemical structure	 *denotes the position of the ^{14}C-radiolabel
Radiolabelled test material	[Pyrazole-4-^{14}C]BCS-CN88460
Specific radioactivity	4.22 MBq/mg (113.92 µCi/mg) for test 1, test 2, test 5, test 6 and test 7; 3.9 MBq/mg (105.34 µCi/mg) for test 3 and test 4
Radiochemical purity	> 98% for all aliquots of the test compound certified by HPLC and TLC (radio-detection)
Chemical purity	>98% (HPLC)
Non-radiolabelled test material	BCS-CN88460
Chemical purity	98.4%

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Vehicle	0.5% aqueous Tragacanth solution
Preparation of dosing solution	Radiolabelled test compound suspended aqueous Tragacanth solution; test 3 and 4 (high dose): the radiolabelled test compound was diluted with a mixture of non-radiolabelled test compound. The dilution was performed in a ratio of 1 to 99.

2. Test animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	Wistar Hsd/Cpb: WU
Breeding facility:	[redacted]
Sex and numbers involved:	Test 1: 4 male rats Test 2: 4 female rats Test 3: 4 male rats Test 4: 4 female rats Test 5: 4 male rats Test 6: 6 male rats Test 7: 8 female rats
Age:	6 to 7 weeks for male rats and 8 to 9 weeks for female rats at the time of delivery
Body weight:	Males of Test 1: 194 - 207 g at the time of administration 201 - 219 g at the time of sacrifice Females of Test 2: 193 - 207 g at the time of administration 183 - 205 g at the time of sacrifice Males of Test 3: 207 - 212 g at the time of administration 186 - 197 g at the time of sacrifice Females of Test 4: 185 - 190 g at the time of administration 177 - 187 g at the time of sacrifice Males of Test 5: 194 - 201 g at the time of administration 189 - 195 g at the time of sacrifice Males of Test 6: 197 - 213 g at the time of administration 197 - 205 g at the time of sacrifice Females of Test 7: 188 - 203 g at the time of administration 156 - 200 g at the time of sacrifice
Acclimatization:	The animals were acclimated to the laboratory conditions in Makrolon cages on wood shavings in the test facility for about 1 week prior administration.
Identification:	The animals were identified by cage cards listing the study number, test compound name and individual animal number. They were additionally labelled with water-insoluble spots on the tail.
Housing:	The animals were kept under conventional hygienic conditions in air-conditioned rooms. After administration of the radiolabelled test compound, the animals were kept individually in Makrolon metabolism cages. With these cages, an almost quantitative and separate collection of urine and faeces was possible. Temperature 20 - 26 °C, relative humidity 24 - 89 %. 12 / 12 hours light / dark cycle.

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Feed and water:	The rats were fed *ad libitum* with rat/mice maintenance long life diet (V1574-000 Ered I or V1534-000, R/M-H, 10 mm, supplied by [redacted]). They received approximately 16 g to 20g per animal and day. The day before dosing they were fed at approximately 8 am and 4 pm, each point in time with approximately 8 g per animal. Tap water from municipal water supply, *ad libitum*

B. Study design

1. Dosing

For each administration suspension, an adequate aliquot of the corresponding stock solution was concentrated to near dryness under a gentle stream of nitrogen. For the high-dose experiments (test 3 and 4), the radiolabelled test compound was radio diluted with the non-radiolabelled test compound. The radio dilution was performed in a ratio of 1:99.

For each test, the individual portions of test compound were formulated with 0.5% aqueous Tragacanth using an ultrasonic bath. Afterwards the individual administration suspensions were stirred on a magnetic stirrer overnight at approx. 5°C and at room temperature during the administration process.

For test 5, male rats were pre-treated with an administration suspension containing non-radiolabelled test compound. The non-radiolabelled test compound was formulated with 0.5% Tragacanth as described above.

After each radioactive dosing of rats, the identity and the radiochemical purity of the test compound in the administration suspension was confirmed by HPLC analysis.

Rats were orally administered by gavage using a syringe attached to an animal-feeding knob cannula.

The concentration of each administration suspension was calculated to achieve an administered amount of about 2 mg test compound per kg body weight (bw) for test 1, 2, 5, 6 and 7 and about 200 mg/kg bw for test 3 and 4, assuming an average animal weight of approximately 200 g at the time of dosing (see table below). After administration of the radiolabelled test compound the rats were kept individually in Makrolon metabolism cages, which allowed separate and quantitative collection of urine, bile, and faeces. Plasma, urine, bile, and faeces samples were collected at various times separately for each animal. Organs and tissues were dissected from the animals at sacrifice. The collection intervals for the respective samples are summarized in the table below.

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Table 5.1.1- 2: Experiments conducted in the ADME study with [pyrazole-4-^{14}C]BCS-CN88460

Test ID	Test description	Time of sample collection				
		Urine [h]	Faeces [h]	Bile [h]	Organs [h]	Microplasma [h]
1	4 male rats 2 mg/kg bw single oral low dose	4, 8, 12, 24, 48, 72	24, 48, 72	---	72	
2	4 female rats 2 mg/kg bw single oral low dose	4, 8, 24, 48, 72	24, 48, 72	---	72	0.25, 0.5, 1, 2, 4, 24, 48, 72
3	4 male rats 200 mg/kg bw single oral high dose	4, 8, 24, 48, 72	24, 48, 72	---	72	
4	4 female rats 200 mg/kg bw single oral high dose	4, 8, 24, 48, 72	24, 48, 72	---	72	
5	4 male rats 2 mg/kg bw low dose pre-treatment with non-radioactive test compound for 14 days plus a single radioactive low dose	4, 8, 24, 48, 72	24, 48, 72	---	72	0.25, 0.5, 1, 2, 4, 7, 24, 48, 72
6	6 bile-duct cannulated male rats * 2 mg/kg bw single oral dose	4, 8, 24, 48	24, 48	4, 8, 24, 32, 48	48	---
7	8 bile-duct cannulated female rats ** 2 mg/kg bw single oral dose	4, 8, 24, 48	24, 48	4, 8, 24, 32, 48	48	---

* Two animals were sacrificed approx. 24 h after treatment, due to no bile was collected.
** Four animals were sacrificed during the test or not used for evaluation.

2. Sample collection

Collection of plasma

Blood samples were collected separately as micro sample for each animal by pressing a capillary coated with heparin in a small cut in the tail vein. The wound was closed with adhesive tape. The capillaries were centrifuged at approximately 12000 g for 10 minutes using a haematocrit centrifuge to separate plasma from the blood cells. This method allows to collect plasma samples at different time points from the same animals and to generate plasma curves from single animals. This procedure results in lower variability in contrast to curves calculated from whole blood samples of different animals and therefore avoids inter-animal variation.

Collection of excreta

Immediately after the administration of the test compound, animals were transferred to metabolism cages. Urine and faeces were collected separately and quantitatively for each animal. Urine was collected in a cryogenic trap with dry ice. The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction, weighted and radioactivity was determined by LSC (liquid scintillation counting).

Collected faeces were weighed, diluted with water in a ratio of about 1:1 (w/v), homogenised before aliquots were taken for the determination of radioactivity by combustion/LSC.

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Collection of bile

A bile-duct cannulation experiment was performed with 6 male and 8 female rats (tests 6 and 7). Bile was collected at various times separately for each animal in cryogenic traps cooled with dry ice. The radioactivity was determined by LSC.

Sacrifice and organ/tissue sampling

All animals were sacrificed in anaesthesia by transection of the cervical blood vessels at 72 hrs after dosing to obtain blood, organs and tissues. Only in the bile duct cannulation test the animals were sacrificed after 48 hrs. The blood collected at sacrifice was separated into plasma and erythrocytes by centrifugation. The organs and tissues were dissected, weighed immediately and lyophilised. Finally, they were homogenised prior aliquots were taken for the determination of radioactivity by combustion/LSC. For small organs and tissues such as adrenal glands, thyroid, ovaries, renal fat and uterus, only the wet weight was determined before the organs were solubilised followed by radio assay by LSC. The radioactivity remaining in the GIT, skin and residual carcass was determined by combustion/LSC in order to establish the radioactivity balance.

3. Measurement of radioactivity

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

4. Toxicokinetic analysis

In this study, the software TOPFIT version 2.0 was used to calculate toxicokinetic parameters by plasma concentration-time curve analysis for the mean dose normalised equivalent concentration-values from each test. A standard 2-compartment disposition model was applied for curve fitting computation. Compartments are defined as physical locations in the body that can be represented with certain simplifications during modelling. Compartment models attempt to describe the following processes mathematically: absorption of the administered compound, entry into the systemic circulation, distribution into organs or tissues where metabolism can occur, and subsequent excretion.

A list of the toxicokinetic parameters that is addressed in this study is given in the table below.

$AUC_{0-\infty}$	Total area under the plasma radioactivity concentration-time curve extrapolated from time 0 to infinity [mg/kg x h for "C"; g/g x h for "C_{norm}"]
$t_{1/2\ e1}$	Half-life of the distribution and elimination phase [h]
$t_{1/2\ e2}$	Half-life of the terminal elimination phase [h]
t_{max}	Time at which the maximum radioactivity concentration occurs in plasma following administration of an extravascular dose [h]
C_{max}	Maximum radioactivity concentration observed in plasma following administration of an extravascular dose [mg/kg for "C"; g/g for "C_{norm}"]

5. Metabolite analysis

For analysis and quantification of the parent compound and metabolites in urine, bile, and faeces, respective samples from all animals of a test were pooled, and samples from these pools were used for characterisation, identification and quantification.

Urine and bile samples were analysed directly without a preceding sample preparation by HPLC based on reversed phase chromatography using an acidic water/acetonitrile/tetrahydrofurane gradient. Pooled

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faeces samples were conventionally and exhaustively extracted with mixtures of acetonitrile, water and formic acid. Prominent metabolites were identified in isolated fractions from the urine (test 1, 0 - 4 h), in isolated fractions from the bile of test 6 (0 - 32 h) and in isolated fractions of feaces from the conventional extract of test 2 and 4 (both 0 - 48 h). The fractions were purified using different column types and solvent gradients. The metabolites in the fractions were identified based on spectroscopic methods, co-chromatography with radio-labelled reference compounds or assignment of the retention times. In addition, all bile samples were enzymatically cleaved with ß-glucuronidase/arylsulfatase for 4 days at 37°C. The cleavage solution was concentrated, purified and analysed by HPLC. From these HPLC analyses the cleavage rates of BCS-CN8860-2-propanol and BCS-CN88460-desmethyl-propanol were determined.

Identical metabolites in urine, bile, and extract of faeces of the current study were assigned to each other based on their retention times and their spectroscopic behaviour and were named with the same report name. Identical identified metabolites in the current study and the ADME study with the phenyl label were also named with the same report name. The assignment of these metabolites in the profiles was achieved by comparison of the retention times and metabolite profiles.

An overview on the reference compounds is given in Table 5.1.1-3.

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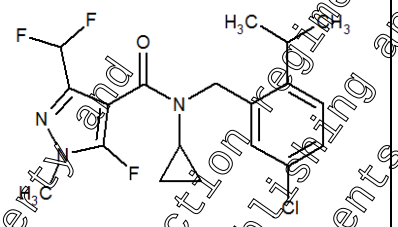
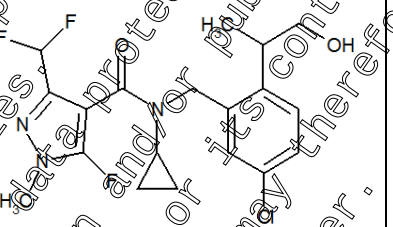
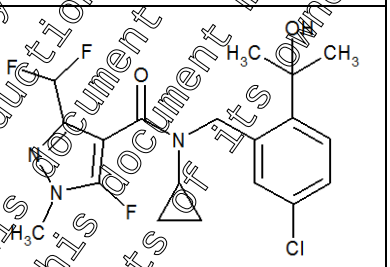
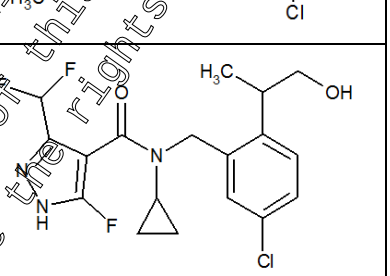
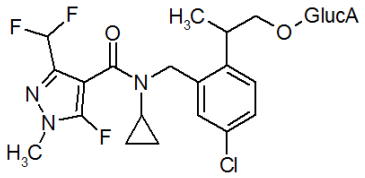
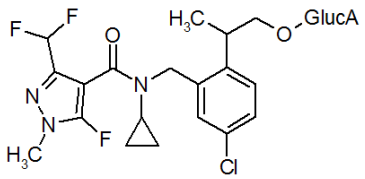
Table 5.1.1- 3: List of reference compounds

Report name/ BCS codes	**Chemical name (IUPAC)**	**Chemical Structure**
Parent compound **BCS-CN88460** (non-radiolabelled and radiolabelled)	N-(5-chloro-2-isopropylbenzyl)-N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazole-4-carboxamide	
BCS-CN88460-propanol BCS-CY24813 sample ID: BN4183B	N-[5-chloro-2-(1-hydroxypropan-2-yl)benzyl]-N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazole-4-carboxamide	
BCS-CN88460-2-propanol BCS-DC20298 sample ID: YK0614H1	N-[5-chloro-2-(2-hydroxypropan-2-yl)benzyl]-N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazole-4-carboxamide	
BCS-CN88460-desmethyl-propanol BCS-DC22055 sample ID: YK0614E1	N-[5-chloro-2-(1-hydroxypropan-2-yl)benzyl]-N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1H-pyrazole-4-carboxamide	
BCS-CN88460-propanol-GlucA (isomer 1) sample ID: YK0413B3	2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazol-4-yl]carbonyl}amino)methyl]phenyl}propyl glucopyranosiduronic acid	
BCS-CN88460-propanol-GlucA (isomer 2) sample ID: YK0413D3	2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazol-4-yl]carbonyl}amino)methyl]phenyl}propyl glucopyranosiduronic acid	

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II. RESULTS AND DISCUSSION

A. Recovery

In general, between 100.3% (test 2) and 104.5% (test 7) of the administered dose were recovered as shown by measurements of radioactivity in urine, bile, faeces, and in organs and tissues at sacrifice. A summary of the radioactivity in percent of the administered dose found in excreta and organs and tissues at sacrifice is presented in the tables below.

Table 5.1.1- 4: Recovery of radioactivity in excreta, gastrointestinal tract and the body of rats following oral dosing of [pyrazole-4-^{14}C]BCS-CN88460

Percent of total radioactive dose administered (mean values)

Test no.	**Test 1**	**Test 2**	**Test 3**	**Test 4**	**Test 5**	**Test 6**	**Test 7**
Experiment	**male** single low dose	**female** single low dose	**male** single high dose	**female** single high dose	**male** pre-treatment	**male** bile-duct cannulation	**female** bile-duct cannulation
Dose	2 mg/kg bw	2 mg/kg bw	200 mg/kg bw	200 mg/kg bw	2 mg/kg bw	2 mg/kg bw	2 mg/kg bw
Urine	14.06	12.00	7.11	8.51	12.67	5.89	2.37
Bile	---	---	---	---	---	77.61	85.45
Faeces	89.28	88.14	93.44	92.03	90.01	20.67	15.52
Total excreted	**103.34**	**100.13**	**100.55**	**100.54**	**102.68**	**104.16**	**103.34**
Body excluding GIT	0.379	0.172	0.153	0.155	0.294	0.252	0.218
GIT	0.030	0.016	0.017	0.012	0.045	n.c.	0.980
Total in body	0.409	0.188	0.170	0.167	0.339	0.306	1.198
Balance	**103.75**	**100.32**	**100.72**	**100.71**	**103.02**	**104.47**	**104.54**

Percent of total radioactive dose recovered (mean values)

Test no.	**Test 1**	**Test 2**	**Test 3**	**Test 4**	**Test 5**	**Test 6**	**Test 7**
Experiment	**male** single low dose	**female** single low dose	**male** single high dose	**female** single high dose	**male** pre-treatment	**male** bile-duct cannulation	**female** bile-duct cannulation
Dose	2 mg/kg bw	2 mg/kg bw	200 mg/kg bw	200 mg/kg bw	2 mg/kg bw	2 mg/kg bw	2 mg/kg bw
Urine	13.57	11.98	7.06	8.44	12.51	5.60	2.26
Bile	---	---	---	---	---	74.10	81.64
Faeces	86.03	87.83	92.77	91.39	87.36	20.00	14.94
Total excreted	**99.61**	**99.81**	**99.83**	**99.83**	**99.67**	**99.71**	**98.84**
Body excluding GIT	0.365	0.172	0.152	0.153	0.286	0.241	0.209
GIT	0.029	0.016	0.017	0.012	0.044	n.c.	0.952
Total in body	0.394	0.188	0.169	0.165	0.329	0.295	1.162
Norm. - factor	0.964	0.997	0.993	0.993	0.971	0.959	0.957
Absorption rate [%]:						**79.9**	**84.1**

n.c. - not calculated
--- - no sample collected

B. Absorption

The absorption of BCS-CN88460 started immediately after oral administration as shown by the mean measured concentration of radioactivity in the plasma of the low dose and high dose tests (2 mg/kg bw and 200 mg/kg bw, respectively). For all low dose tests the mean maximum plasma concentration (C_{max}) was reached at 1 h (t_{max}) after administration and ranged from 0.8497 µg/g to 1.3467 µg/g.

The time course of the mean plasma levels was comparable in male and female rats.

In the high dose tests, the mean maximum plasma concentration was measured at 2 h after dosing for male rats (test 3) and at 4 h after dosing for female rats (test 4). The highest mean equivalent plasma concentration amounted to 30.08 µg/g in male rats and to 27.02 µg/g in female rats.

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Radioactivity could be detected in all plasma samples until 72 h after dosing, the latest time of plasma sampling, with values ranging from approximately 0.2% (test 1) to 3.1% (test 4) of the maximum plasma concentration measured (calculated from values of the tables below).

Low dose tests with bile duct cannulated male and female rats showed that about 74% of the recovered dose was detected in the bile of male animals (test 6) and about 82% in the bile of females (test 7). Absorption rates were calculated by summation of the recovered radioactivity in urine, bile, and body without GIT and amounted to 79.9% in males and 84.1% in females.

Table 5.1.1- 5: Time course of radioactivity in the plasma of male and female rats following an oral dose of [pyrazole-4-^{14}C]BCS-CN88460

Equivalent concentration [mg active substance equivalent / kg $_{sample}$] (mean values)

Time [h post admin.]	Test 1 male 2 mg/kg bw	Test 2 female 2 mg/kg bw	Test 3 male 200 mg/kg bw	Test 4 female 200 mg/kg bw	Test 5 pre-treatment male 2 mg/kg bw
0.25	0.2706	0.4261	2.7569	1.9249	0.6567
0.5	0.6586	0.6907	8.5666	4.8523	1.2380
1	0.8497	0.9316	17.2689	10.3262	1.3467
2	0.6382	0.7879	30.0761	16.6495	0.7222
4	---	---	---	27.0158	0.4227
7	0.2030	0.3196	27.2620	26.1512	0.2902
24	0.0346	0.0295	1.8094	2.3717	0.0408
48	0.0292	0.0211	0.8067	1.1493	0.0210
72	0.0156	0.0091 *	0.5050	* 0.8276	0.0161

Dose normalised concentration CN (mean values)

Time [h post admin.]	Test 1 male 2 mg/kg bw	Test 2 female 2 mg/kg bw	Test 3 male 200 mg/kg bw	Test 4 female 200 mg/kg bw	Test 5 pre-treatment male 2 mg/kg bw
0.25	0.1565	0.2203	0.0142	0.0087	0.2809
0.5	0.3828	0.3560	0.0441	0.0219	0.5284
1	0.4931	0.4793	0.0890	0.0466	0.5735
2	0.3703	0.4050	0.1550	0.0752	0.3078
4	---	---	---	0.1221	0.1803
7	0.1180	0.1645	0.1406	0.1184	0.1236
24	0.0201	0.0152	0.0093	0.0107	0.0174
48	0.0117	0.0110	0.0042	0.0052	0.0090
72	0.0090	0.0047 *	0.0026	* 0.0038	0.0069

--- - no sample collected
* - mean value calculated with half of LLQ

C. Distribution and plasma kinetics

The distribution of the test substance and its metabolites from the central compartment (blood plasma) into the different organs and tissues was investigated by measuring the concentration of the total radioactivity in plasma.

After a single oral administration of the low dose [pyrazole-4-^{14}C]BCS-CN88460 to male and female rats, the mean maximum plasma concentration of the radioactivity was measured at 1 h post administration (t_{max}). In the high dose tests, the measured maximum of the plasma concentration was

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reached at 2 h for male rats and at 4 h for female rats. In all low dose tests the maximum dose normalised concentration was in the range from 0.479 to 0.574. In the high dose test the maximum dose mean normalised concentration amounted to 0.122 in female rats and to 0.155 in male rats. The lower values after administration of the high dose are assumed to be due to lower absorption behaviour of the test compound compared to the low dose tests.

For all tests the plasma concentration declined to values below 3.1% of the maximum concentration within 72 h post administration. This indicates no retention of compound related residues in the body of the animals.

The plasma concentration in the low dose tests as well as the high dose tests were calculated with a two-compartment model by TOPFIT. The weighting of $1/y^2$ was used due to a fast elimination phase after reaching the plasma peak followed by a slower elimination phase after approximately 24 h.

There were no sex specific differences in the calculated $AUC_{0-\infty}$ values for low or high dose male and female rats. However, rats dosed with 2 mg/kg bw showed an approximately 1.75-times higher exposure compared to rats dosed with 200 mg/kg bw, due to the lower absorption at higher dose levels.

Table 5.1.1- 6: Pharmacokinetic parameters of [pyrazole-4-^{14}C]BCS-CN88460 after oral administration to male and female rats, derived from plasma curve analysis

Nominal dose (mg/kg bw)		**Test 1** **male** **2**	**Test 2** **female** **2**	**Test 3** **male** **200**	**Test 4** **female** **200**	**Test 5** **male** **2**
Kind of administration		oral	oral	oral	oral	oral after pre-treatment
Actual dose (mg/kg bw)		1.72	1.93	193.46	221.99	2.34
Compartment model		two	two	two	two	two
Weighting of the TOPFIT calculation		$1/y^2$	$1/y^2$	$1/y^2$	$1/y^2$	$1/y^2$
t_{max}	[h] measured	1	1	2	4	1
t_{max}	[h] calculated	0.92	1.27	3.69	4.49	0.48
C_{max}	[µg/mL] measured	0.493	0.479	0.155	0.122	0.574
C_{max}	[µg/mL] calculated	0.468	0.472	0.179	0.109	0.504
$t_{1/2\ e1}$	[h]	0.18	0.36	2.40	2.96	0.05
$t_{1/2\ e2}$	[h]	44.9	31.5	38.6	88.9	37.8
$AUC_{0-\infty}$	[g/g*h]	3.73	3.63	2.13	2.07	3.22

D. Excretion

Generally, in the low and high dose tests the excretion was almost completed 72 h after administration. At this time more than 98% of the recovered dose had been excreted via urine and faeces. In all low and all high dose tests the main portion of radioactivity (>82%) was excreted latest after 24 h (see tables below).

In all tests the excretion was predominantly faecal. Faecal excretion of individual rats ranged from about 83% to 96% of the recovered radioactivity, with exception of the tests with bile-duct cannulated rats. In bile-duct cannulated rats, approximately 20% of the recovered dose (mean value) was detected in faeces of male rats and approximately 15% in faeces of females. Approximately 74% of the mean dose recovered was detected in bile samples of male bile-duct cannulated rats and approximately 82% in bile samples of females.

For single low dose tests the mean urinary excretion rate was 13.6% of the dose recovered for male rats and 12.0% for female rats. For high dose tests mean values of 7.1% of the dose were recovered in

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the urine of male rats and 8.4% of the dose in the urine of female rats. The lower urinary excretion rates at the high dose tests give a hint, that there is a lower absorption of BCS-CN88460 at higher dose rates.

Table 5.1.1- 7: Cumulative excretion of radioactivity at time intervals expressed as % dose administered (mean values)

Time [h post admin.]	Test 1 male oral 2 mg/kg bw	Test 2 female oral 2 mg/kg bw	Test 3 male oral 200 mg/kg bw	Test 4 female oral 200 mg/kg bw	Test 5 male oral pre-treatment 2 mg/kg bw	Test 6 male oral bile-duct cannulation 2 mg/kg bw	Test 7 female oral bile-duct cannulation 2 mg/kg bw
Urine							
4	2.33	2.74	0.58	0.96	5.16	n.c.	0.62
8	7.83	6.33	1.70	2.76	8.78	3.39	1.45
12	10.71	---	---	---	---	---	---
24	13.73	11.58	6.79	8.24	12.34	5.54	2.12
48	14.00	14.93	7.08	8.48	12.61	5.89	2.37
72	14.06	12.00	7.11	8.51	12.67	---	---
Bile							
4	---	---	---	---	---	40.08	35.44
8	---	---	---	---	---	62.60	57.20
24	---	---	---	---	---	76.01	82.25
32	---	---	---	---	---	76.58	84.25
48	---	---	---	---	---	77.61	85.45
Faeces							
24	85.87	83.94	91.39	90.60	84.33	20.24	14.37
48	89.08	88.00	93.30	91.96	89.70	20.67	15.52
72	89.28	88.14	93.44	92.03	90.01	---	---
Sum excreted:	**103.34**	**100.13**	**100.55**	**100.54**	**102.68**	**104.16**	**103.34**

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Table 5.1.1- 8: Cumulative excretion of radioactivity at time intervals expressed as % dose recovered (mean values)

Time [h post admin.]	Test 1 male oral 2 mg/kg bw	Test 2 female oral 2 mg/kg bw	Test 3 male oral 200 mg/kg bw	Test 4 female oral 200 mg/kg bw	Test 5 male oral pre-treatment 2 mg/kg bw	Test 6 male oral bile-duct cannulation 2 mg/kg bw	Test 7 female oral bile-duct cannulation 2 mg/kg bw
Urine							
4	2.24	2.74	0.58	0.95	5.02	n.c.	0.58
8	7.56	6.33	1.68	2.73	8.52	3.21	1.37
12	10.34	---	---	---	---	---	---
24	13.25	11.57	6.75	8.17	11.98	5.27	2.02
48	13.51	11.92	7.03	8.41	12.25	5.60	2.26
72	13.57	11.98	7.06	8.44	12.31	---	---
Bile							
4	---	---	---	---	---	38.42	33.65
8	---	---	---	---	---	59.99	54.50
24	---	---	---	---	---	72.61	78.57
32	---	---	---	---	---	73.14	80.49
48	---	---	---	---	---	74.10	81.64
Faeces							
24	82.77	83.65	90.73	89.98	81.86	19.61	13.85
48	85.84	87.69	92.63	91.32	87.06	20.00	14.94
72	86.03	87.83	92.77	91.39	87.36	---	---
Sum excreted:	**99.61**	**99.81**	**99.83**	**99.83**	**99.67**	**99.71**	**98.84**
Norm. - factor	0.964	0.997	0.993	0.993	0.971	0.959	0.957

E. Residues in organs and tissues at sacrifice

Radioactive residues in tissues and organs at sacrifice (72 h post oral administration of [pyrazole-4-^{14}C]BCS-CN88460) were determined in male and female rats dosed at 2 mg/kg bw (test 1 and 2), 200 mg/kg bw (test 3 and 4), or after repeated dosing of male rats with BCS-CN88460 at 2 mg/kg bw (test 5), respectively. Of bile-duct cannulated male and female rats (at 2 mg/kg bw) only samples of skin and carcass were collected (test 6 and 7).

Generally, there were moderate radioactive residues in organs and tissues of all tests. In the low dose tests, female rats showed lower organ concentration compared to male rats in all tissues except of the perirenal fat. However, amounts of radioactivity were in the same order of magnitude. Following administration of a single high dose, female rats showed slightly higher organ concentrations compared to male rats in all tissues except of the liver. Lower organ concentrations following pre-treatment with BCS-CN88460 than post administration of a single oral dose were observed.

At sacrifice low levels of radioactivity (between 0.152% and 0.365% of the dose (mean values) were found in the bodies excluding GIT. Low amounts of radioactivity were detected in the GITs (0.012% to 0.044 %), excluding GITs from bile-duct cannulated rats. Thus, the elimination of the test compound related radioactivity was nearly completed at sacrifice.

The highest concentration of radioactivity was detected in the liver of all low dose and all high dose tests. The equivalent concentration ranged from 0.0338 to 3.5395 mg/kg. The concentration in blood

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cells was high compared to the concentrations in organs and tissues excluding liver. It ranged from 0.0197 to 2.4061 mg/kg. The mean concentration in the other organs and tissues ranged from 0.0009 to 1.6321 mg/kg.

From the elimination kinetics of the total radioactivity from plasma it can be concluded that small amounts of residual radioactivity in organs and tissues are subject to further elimination.

Table 5.1.1- 9: Radioactive residues in organs and tissues at sacrifice expressed as equivalent concentration [mg active substance equivalent / kg sample] (mean values)

Organs/ Tissues	Test 1 male 2 mg/kg bw	Test 2 female 2 mg/kg bw	Test 3 male 200 mg/kg bw	Test 4 female 200 mg/kg bw	Test 5 pre-treatment male 2 mg/kg bw
Blood Cells	0.0294	0.0197	1.7741	2.4061	0.0245
Plasma	0.0147	0.0073	0.5330	1.6321	0.0143
Carcass	0.0027	0.0019	0.1543	0.2153	0.0029
Heart	0.0052	0.0030	0.2646	0.3904	0.0062
Brain	0.0014	0.0009 *	0.0739	0.1208	0.0014
Kidneys	0.0110	0.0067	0.5642	0.5803	0.0129
Liver	0.0750	0.0338	3.5395	2.6915	0.1002
Testes	0.0025	---	0.1301	---	0.0026
Ovaries	---	0.0041	---	* 0.3460	---
Uterus		0.0035	---	n.c.	---
Adrenal gland	0.0057	0.0045 *	0.3710	0.4465	0.0067
Thyroid gland	n.c.	n.c.	n.c.	n.c.	n.c.
Spleen	0.0051	0.0043	0.3456	0.4539	0.0065
Lung	0.0089	0.0043	0.4484	0.6743	0.0103
Eyes	0.0018	0.0016	0.1029	0.1454	0.0016
Skin	0.0038	0.0026	0.2142	* 0.2234	0.0039
Bone femur	0.0021	n.c.	n.c.	n.c.	0.0026
Perirenal fat	0.0066	0.0073	0.3768	0.5480	0.0059
Muscle leg	0.0020	0.0012	0.1258	0.1438	0.0019

n.c. - not calculated
--- - no sample collected
* - mean value calculated with half of LLQ

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Table 5.1.1- 10: Radioactive residues in organs and tissues at sacrifice expressed as dose normalised concentration (mean values)

Organs/ Tissues	Test 1 male 2 mg/kg bw	Test 2 female 2 mg/kg bw	Test 3 male 200 mg/kg bw	Test 4 female 200 mg/kg bw	Test 5 pre-treatment male 2 mg/kg bw
Blood Cells	0.0174	0.0096	0.0084	0.0106	0.0101
Plasma	0.0088	0.0036	0.0025	0.0072	0.0059
Carcass	0.0016	0.0009	0.0007	0.0009	0.0012
Heart	0.0031	0.0014	0.0012	0.0017	0.0026
Brain	0.0008	0.0004	* 0.0003	0.0005	0.0006
Kidneys	0.0065	0.0033	0.0027	0.0026	0.0054
Liver	0.0448	0.0165	0.0167	0.0118	0.0416
Testes	0.0015	---	0.0006	---	0.0011
Ovaries	---	0.0020	---	0.0015	---
Uterus	---	0.0017	---	n.c.	---
Adrenal gland	0.0034	0.0022	* 0.0018	0.0020	0.0028
Thyroid gland	n.c.	n.c.	n.c.	n.c.	n.c.
Spleen	0.0030	0.0021	0.0016	0.0020	0.0027
Lung	0.0053	0.0021	0.0021	0.0030	0.0043
Eyes	0.0011	0.0008	0.0005	0.0006	0.0007
Skin	0.0023	0.0013	0.0010	* 0.0016	0.0016
Bone femur	0.0013	n.c.	n.c.	n.c.	0.0011
Perirenal fat	0.0039	0.0035	0.0018	0.0024	0.0024
Muscle leg	0.0012	0.0006	0.0006	0.0006	0.0008

n.c. - not calculated
--- - no sample collected
* - mean value calculated with half of LLQ

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Table 5.1.1- 11: Radioactive residues in organs and tissues at sacrifice expressed as % dose administered

Organs/ Tissues	Test 1 male 2 mg/kg bw	Test 2 female 2 mg/kg bw	Test 3 male 200 mg/kg bw	Test 4 female 200 mg/kg bw	Test 5 pre-treatment male 2 mg/kg bw
Blood Cells**	0.0246	0.0133	0.0137	0.0145	0.0142
Plasma**	0.0117	0.0046	0.0034	0.0091	0.0053
Carcass	0.0808	0.0523	0.0387	0.0488	0.0650
Heart	0.0011	0.0006	0.0005	0.0007	0.0010
Brain	0.0007	0.0004	0.0003 *	0.0005	0.0005
Kidneys	0.0053	0.0026	0.0023	0.0020	0.0045
Liver	0.1969	0.0659	0.0675	0.0568	0.1597
Testes	0.0020	---	0.0008	---	0.0018
Ovaries	---	0.0001 *	---	0.0001 *	---
Uterus	---	0.0005	---	n.c.	---
Adrenal gland	0.0001	0.0001	0.0001	0.0001	0.0001
Thyroid gland	n.c.	n.c.	n.c.	n.c.	n.c.
Spleen	0.0006	0.0004	0.0003	0.0005	0.0005
Lung	0.0032	0.0013	0.0013	0.0019	0.0035
Eyes	0.0001	0.0001	0.0001	0.0001	0.0001
Skin	0.0494	0.0279	0.0232 *	0.0195	0.0365
Bone femur**	0.0003	n.c.	n.c.	n.c.	0.0004
Perirenal fat**	0.0007	0.0014	0.0003	0.0005	0.0002
Muscle leg**	0.0011	0.0005	0.0005	0.0006	0.0008

n.c. - not calculated
--- - no sample collected
* - mean value calculated with half of LLQ
** - Of these organs or tissues only an aliquot was sampled at sacrifice, the percentage of dose administered is relating to the aliquot of organ/tissue sampled and analysed. The contribution of the part which was not sampled is included in the value for the carcass.

F. Metabolism

For investigation of the metabolism, urine, bile and faeces were sampled at various time points during the individual tests.

Urine and bile samples were analysed without any additional sample preparation. In all tests faeces were conventionally extracted with a mixture of acetonitrile/water (8/2, v/v) plus formic acid, followed by an exhaustive extraction with acetonitrile/water (1/1; v/v) and acetonitrile/water (1/1; v/v) plus 3% formic acid using microwave assistance for test 1, test 2 and test 5. The conventional extraction rates ranged from 93.8% to 97.9% of the radioactivity in faeces. After exhaustive extraction, between 0.35% and 2.59% of the administered dose was detected in the post extraction solids (PES) only. There were no losses during sample preparation.

Parent compound and metabolites were analysed and quantified in urine, bile and extracts of faeces by radio-HPLC. The metabolite pattern in the urine samples and extracts of faeces from the current study and the ADME study with the phenyl-label ([redacted], R.; [redacted], N.; 2017; M-602883-02-1) were similar, except of the individual label specific metabolites. Corresponding metabolites in urine, bile and faeces extracts from all ADME rat studies on BCS-CN88460 were assigned, depending on their retention times based on the profiling HPLC method.

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Metabolites were identified in isolated fractions from urine, bile, and extracts of faeces by spectroscopic methods. BCS-CN88460-desmethyl-propanol-GlucA (isomer 1 and 2) and BCS-CN88460-propanol-GlucA (isomer 1 and 2) were identified based on enzymatic cleavage of the bile sample and/or HPLC co-chromatography with radiolabelled reference compounds. In addition metabolites were identified in the course of the ADME study with the phenyl label ([redacted], R.; [redacted], N.; 2017; M-602883-02-1) and assigned in the profiles by comparison of the retention times and metabolite pattern.

Metabolite profiles of the low dose tests showed more metabolic transformation compared to the high dose tests. In the high dose tests parent compound was excreted exclusively via faeces and amounted to more than 57% of the radioactivity excreted. Qualitative and quantitative differences were observed in the metabolic profiles of male and female rats in the low and high dose tests. A high number of various metabolites was characterised and identified in samples of male and female rats. No relevant qualitative or quantitative differences in the metabolic profile of pre-treated male rats were observed in comparison to single dosing.

The identification rates of parent compound and metabolites were high and amounted to between 85.61% and 100.79% of the administered dose in all tests.

Parent compound and metabolites

Parent compound was detected between 0.73% and 4.86% of the dose in the low dose tests including pre-treatment, except bile-duct cannulation. Parent compound amounted to 18.24% of dose for bile-duct cannulated males and 15.56% of dose for bile-duct cannulated females. For high dose tests 57.26% of dose (males) and 59.07% of dose were identified as parent compound. Parent compound was not found in the urine.

Major metabolites in male and female rats were BCS-CN88460-carboxylic acid (up to 14.34% of dose for males, pre-treatment) and BCS-CN88460-desmethyl-carboxylic acid (up to 18.84% of dose for females), except in bile-duct cannulated rats. BCS-CN88460-desmethyl-hydroxyphenyl-1,2-propandiol (up to 8.11% of dose), BCSCN88460-desmethyl-OH-GlucA (isomer 2) (approx. 10% of dose) were found in low dose males (single and pre-treatment). BCS-CN88460-desmethyl, BCS-CN88460-desmethyl-hydroxymethyl-carboxylic acid and BCS-CN88460-desmethyl-propanol were found in low dose female rats and amounted to 11.43% of dose, 11.38% of dose and 8.68 % of dose, respectively. BCS-CN88460-desmethyl-GlucA (isomer 1 and 2) (14.09% and 7.26% in females, respectively), BCS-CN88460-propanol-GlucA (isomer 1) (6.98% in males and 12.27% in females) and BCS-CN88460-desmethyl-diOH-GlucA (isomer 1) (5.56% of dose in males) were major conjugates in excreta of bile-duct cannulated rats.

Prominent metabolites were BCS-CN88460-cyclopropyl-pyrazole-carboxamide-GlucA (isomers), BCS-CN88460-pyrazole-carboxylic acid-Ala, BCS-CN88460-desmethyl-hydroxymethyl-diOH, BCS-CN88460-desmethyl-diOH-GlucA (isomer 4) and (isomer 5), BCS-CN88460-desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2), BCS-CN88460-desmethyl-hydroxyphenyl-2-propanol, BCS-CN88460-desmethyl-lactic acid, BCS-CN88460-lactic acid, BCS-CN88460-propanol-GlucA (isomer 2), BCS-CN88460-propanol and BCS-CN88460-desmethyl-propanol-GlucA (isomer 2). All these metabolites amounted to above 2% of dose, but not more than 6.74% of dose.

A high number of metabolites amounted to < 2.0% of dose and were identified as BCS-CN88460-pyrazole-amide, BCS-CN88460-cyclopropyl-pyrazole-carboxamide-OH-GlucA, BCS-CN88460-pyrazole-carboxylic acid, BCS-CN88460-desfluoro-N-methyl-pyrazole-carboxylic acid, BCS-CN88460-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys, BCS-CN88460-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys-Gly, BCS-CN88460-N-methyl-pyrazole-carboxylic acid, BCS-CN88460-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH, BCS-CN88460-cyclopropyl-pyrazole-carboxamide, BCS-CN88460-cyclopropyl-oxy-pyrazole-carboxamide, BCS-CN88460-desmethyl-triOH-GlucA, BCS-CN88460-desmethyl-diOH-GlucA (isomer 2 and isomer 3), BCS-CN88460-desmethyl-OH-GlucA (isomer 1), BCS-CN88460-desmethyl-diOH (isomer), BCS-CN88460-desmethyl-oxo-GlucA, BCS-CN88460-carboxylic acid-GlucA, BCS-CN88460-desmethyl-carboxylic acid-GlucA (isomer 2), BCS-CN88460-desmethyl-

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propanol-GlucA (isomer 1), BCS-CN88460-olefine, BCS-CN88460-oxo-GlucA, BCS-CN88460-lactic acid, BCS-CN88460-desmethyl-diOH-GlucA (isomer 6), BCS-CN88460-propanol-GlucA (isomer 1 and 2), BCS-CN88460-desmethyl-SA and BCS-CN88460-2-propanol.

More metabolites may be present as indicated by broad non-resolved zones in the chromatograms. Unidentified metabolites were characterised by their extraction and chromatographic behaviour.

All results correspond well with the findings in the ADME study with the phenyl label.

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Table 5.1.1- 12: Test 1: Balance of BCS-CN88460 and metabolites excreted after oral administration of 2 mg/kg bw to male rats expressed as % dose administered

Report name BCS-CN88460-	Test 1 (male, 2 mg/kg bw)		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	Total
pyrazole-amide	0.86	---	0.86
cyclopropyl-pyrazole-carboxamide-OH-GlucA	0.72	---	0.72
pyrazole-carboxylic acid	1.59	---	1.59
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)	2.85	---	2.85
desfluoro-N-methyl-pyrazole-carboxylic acid	0.50	---	0.50
pyrazole-carboxylic acid-Ala	4.92	---	4.92
N-methyl-pyrazole-carboxylic acid	0.67	---	0.67
cyclopropyl-pyrazole-carboxamide	0.76	---	0.76
cyclopropyl-oxy-pyrazole-carboxamide	0.31	---	0.31
desmethyl-triOH-GlucA	---	0.98	0.98
desmethyl-diOH-GlucA (isomer 1)	---	1.12	1.12
desmethyl-hydroxymethyl-diOH	---	2.86	2.86
desmethyl-diOH-GlucA (isomer 3)	---	0.52	0.52
desmethyl-hydroxyphenyl-1,2-propandiol	---	8.11	8.11
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	0.17	1.64	1.81
desmethyl-OH-GlucA (isomer 2)	---	10.24	10.24
desmethyl-hydroxyphenyl-2-propanol	0.13	4.19	4.32
desmethyl-lactic acid	---	1.98	1.98
desmethyl-hydroxymethyl-carboxylic acid	---	3.01	3.01
desmethyl-diOH-isomer)	---	1.57	1.57
lactic acid	---	2.67	2.67
propanol-GlucA (isomer 1 and 2)	---	1.17	1.17
desmethyl-SA	---	0.28	0.28
desmethyl-carboxylic acid	---	12.44	12.44
desmethyl-propanol	---	1.60	1.60
carboxylic acid	---	12.61	12.61
propanol	---	0.95	0.95
2-propanol	---	0.70	0.70
desmethyl	---	1.44	1.44
parent compound	---	4.11	4.11
Total identified	**13.46**	**74.20**	**87.66**
Total characterised	**0.54**	**9.33**	**9.87**
Number of characterised metabolites (maximum value)	11 (0.19)	11 (2.02)	21 (2.02)
Exhaustive extract of faeces	---	2.98	2.98
Solids of faeces (PES)	---	2.58	2.58
Urine or faeces not analysed (48 - 72 h)	0.06	0.20	0.26
Total			**103.34**

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Table 5.1.1- 13: Test 2: Balance of BCS-CN88460 and metabolites excreted after oral administration of 2 mg/kg bw to female rats expressed as % dose administered

Report name BCS-CN88460-	Test 2 (female, 2 mg/kg bw)		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	Total
pyrazole-amide	0.08	---	0.08
cyclopropyl-pyrazole-carboxamide-OH-GlucA	0.08	---	0.08
pyrazole-carboxylic acid	0.18	---	0.18
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)	0.80	---	0.80
pyrazole-carboxylic acid-Ala	1.69	---	1.69
N-methyl-pyrazole-carboxylic acid	0.37	---	0.37
cyclopropyl-pyrazole-carboxamide	0.60	---	0.60
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	---	1.16	1.16
desmethyl-OH-GlucA (isomer 2)	0.23	---	0.23
desmethyl-hydroxyphenyl-2-propanol	1.29	5.45	6.74
desmethyl-hydroxymethyl-carboxylic acid	0.80	10.58	11.38
desmethyl-carboxylic acid	4.37	14.47	18.84
desmethyl-propanol	---	8.68	8.68
carboxylic acid	0.80	12.98	13.77
propanol	---	4.09	4.09
2-propanol	---	0.64	0.64
desmethyl	---	11.43	11.43
parent compound	---	4.86	4.86
Total identified	**11.29**	**74.33**	**85.61**
Total characterised	**0.64**	**9.96**	**10.61**
Number of characterised metabolites (maximum value)	2 (0.37)	8 (3.16)	8 (3.52)
Exhaustive extract of faeces	---	1.86	1.86
Solids of faeces (PES)	---	1.84	1.84
Urine or faeces not analysed (48 - 72 h)	0.07	0.14	0.21
Total			**100.13**

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Table 5.1.1- 14: Test 3: Balance of BCS-CN88460 and metabolites excreted after oral administration of 200 mg/kg bw to male rats expressed as % dose administered

Report name BCS-CN88460-	Test 3 (male, 200 mg/kg bw)		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	Total
pyrazole-amide	0.31	---	0.31
cyclopropyl-pyrazole-carboxamide-OH-GlucA	0.18	---	0.18
pyrazole-carboxylic acid	0.16	---	0.16
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)	0.89	---	0.89
desfluoro-N-methyl-pyrazole-carboxylic acid	0.19	---	0.19
pyrazole-carboxylic acid-Ala	3.10	---	3.10
N-methyl-pyrazole-carboxylic acid	0.30	---	0.30
cyclopropyl-pyrazole-carboxamide	1.63	---	1.63
desmethyl-hydroxyphenyl-1,2-propandiol	---	0.76	0.76
desmethyl-OH-GlucA (isomer 2)	---	2.22	2.22
desmethyl-hydroxyphenyl-2-propanol	---	3.74	3.74
desmethyl-hydroxymethyl-carboxylic acid	---	2.31	2.31
desmethyl-diOH (isomer)	---	0.47	0.47
lactic acid	---	1.18	1.18
desmethyl-carboxylic acid	---	10.23	10.23
desmethyl-propanol	---	1.22	1.22
carboxylic acid	---	5.80	5.80
propanol	---	0.81	0.81
2-propanol	---	0.83	0.83
desmethyl	---	2.74	2.74
parent compound	---	57.26	57.26
Total identified	**6.76**	**89.58**	**96.34**
Total characterised	**0.32**	**1.13**	**1.45**
Number of characterised metabolites (maximum value)	2 (0.17)	3 (0.59)	5 (0.59)
Solids of faeces (PES)	---	2.59	2.59
Urine or faeces not analysed (48 - 72 h)	0.03	0.14	0.17
Total			**100.55**

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Table 5.1.1- 15: Test 4: Balance of BCS-CN88460 and metabolites excreted after oral administration of 200 mg/kg bw to female rats expressed as % dose administered

Report name BCS-CN88460-	Test 4 (female, 200 mg/kg bw)		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	Total
cyclopropyl-pyrazole-carboxamide-OH-GlucA	0.07	---	0.07
pyrazole-carboxylic acid	0.16	---	0.16
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)	0.53	---	0.53
pyrazole-carboxylic acid-Ala	1.18	---	1.18
N-methyl-pyrazole-carboxylic acid	0.37	---	0.37
cyclopropyl-pyrazole-carboxamide	0.40	---	0.40
desmethyl-OH-GlucA (isomer 2)	---	0.76	0.76
desmethyl-hydroxyphenyl-2-propanol	0.39	1.64	2.03
desmethyl-hydroxymethyl-carboxylic acid	0.49	3.63	4.12
lactic acid	---	0.39	0.39
desmethyl-carboxylic acid	3.63	4.13	7.76
desmethyl-propanol	---	3.34	3.34
carboxylic acid	1.14	5.48	6.62
propanol	---	2.79	2.79
2-propanol	---	0.59	0.59
desmethyl	---	5.31	5.31
parent compound	---	59.07	59.07
Total identified	**8.36**	**87.12**	**95.47**
Total characterised	**0.12**	**2.92**	**3.05**
Number of characterised metabolites (maximum value)	1 (0.12)	4 (1.32)	5 (1.32)
Solids of faeces (PES)	---	1.92	1.92
Urine or faeces not analysed (48 - 72 h)	0.03	0.07	0.10
Total			**100.54**

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Table 5.1.1- 16: Test 5: Balance of BCS-CN88460 and metabolites excreted after oral administration of 2 mg/kg bw to pre-treated male rats expressed as % dose administered

Report name BCS-CN88460-	Test 5 (male, pre-treatment, 2 mg/kg bw)		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	Total
pyrazole-amide	0.50	---	0.50
cyclopropyl-pyrazole-carboxamide-OH-GlucA	0.43	---	0.43
pyrazole-carboxylic acid	1.02	---	1.02
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)	2.36	---	2.36
desfluoro-N-methyl-pyrazole-carboxylic acid	0.41	---	0.41
pyrazole-carboxylic acid-Ala	4.77	---	4.77
N-methyl-pyrazole-carboxylic acid	0.50	---	0.50
cyclopropyl-pyrazole-carboxamide	1.67	---	1.67
cyclopropyl-oxy-pyrazole-carboxamide	0.27	---	0.27
desmethyl-triOH-GlucA	---	0.54	0.54
desmethyl-hydroxymethyl-diOH	---	1.55	1.55
desmethyl-diOH-GlucA (isomer 3)	---	0.31	0.31
desmethyl-hydroxyphenyl-1,2-propanediol	---	6.07	6.07
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	---	1.56	1.56
desmethyl-OH-GlucA (isomer 2)	---	10.47	10.47
desmethyl-hydroxyphenyl-2-propanol	0.22	3.96	4.18
desmethyl-lactic acid	---	1.52	1.52
desmethyl-hydroxymethyl-carboxylic acid	---	6.15	6.15
desmethyl-diOH (isomer)	---	0.55	0.55
lactic acid	---	2.20	2.20
propanol-GlucA (isomer 1 and 2)	---	1.31	1.31
desmethyl-carboxylic acid	---	13.76	13.76
desmethyl-propanol	---	4.43	4.43
carboxylic acid	---	14.34	14.34
propanol	---	2.44	2.44
2-propanol	---	1.19	1.19
desmethyl	---	2.98	2.98
parent compound	---	0.73	0.73
Total identified	**12.13**	**76.04**	**88.17**
Total characterised	**0.48**	**9.55**	**10.03**
Number of characterised metabolites (maximum value)	3 (0.19)	10 (1.96)	13 (1.96)
Exhaustive extract of faeces	---	1.90	1.90
Solids of faeces (PES)	---	2.18	2.18
Urine or faeces not analysed (48 - 72 h)	0.06	0.30	0.36
Fractions not analysed	---	0.04	0.04
Total			**102.68**

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Table 5.1.1- 17: Test 6: Balance of BCS-CN88460 and metabolites excreted after oral administration of 2 mg/kg bw to bile-duct cannulated male rats expressed as % dose administered

Report name BCS-CN88460-	Test 6 (male, bile-duct cannulation, 2 mg/kg bw)			
	Urine (0 - 24 h)	Bile (0 - 32 h)	Faeces (0 - 24 h)	Total
cyclopropyl-pyrazole-carboxamide-OH-GlucA	0.13	0.15	---	0.28
pyrazole-carboxylic acid	0.38	0.36	---	0.74
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)	1.99	1.37	---	3.37
pyrazole-carboxylic acid-Ala	1.50	0.20	---	1.70
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys and desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys-Gly	---	1.57	---	1.57
N-methyl-pyrazole-carboxylic acid	0.26	---	---	0.26
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH	---	0.94	---	0.94
cyclopropyl-pyrazole-carboxamide	0.91	0.09	---	1.00
cyclopropyl-oxy-pyrazole-carboxamide	0.10	---	---	0.10
desmethyl-triOH-GlucA	---	2.80	---	2.80
desmethyl-diOH-GlucA (isomer 1)	---	5.56	---	5.56
desmethyl-diOH-GlucA (isomer 2 and 3)	---	0.85	---	0.85
desmethyl-hydroxymethyl-diOH	---	0.36	---	0.36
desmethyl-diOH-GlucA (isomer 3)	---	1.52	---	1.52
desmethyl-diOH-GlucA (isomer 4)	---	2.17	---	2.17
desmethyl-diOH-GlucA (isomer 5)	---	1.45	---	1.45
desmethyl-OH-GlucA (isomer 1)	---	1.90	---	1.90
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	---	1.64	---	1.64
desmethyl-OH-GlucA (isomer 2)	---	2.09	---	2.09
desmethyl-hydroxyphenyl-2-propanol	0.10	1.30	0.05	1.44
desmethyl-lactic acid	---	3.50	---	3.50
desmethyl-hydroxymethyl-carboxylic acid	---	---	0.20	0.20
desmethyl-diOH (isomer)	---	---	0.06	0.06
desmethyl-oxo-GlucA carboxylic acid-GlucA and desmethyl-carboxylic acid-GlucA (isomer 2)	---	4.58	---	4.58
desmethyl-propanol-GlucA (isomer 1) olefine, oxo-GlucA, lactic acid and desmethyl-diOH-GlucA (isomer 6)	---	13.22	0.02	13.24
propanol-GlucA (isomer 1)	---	6.98	---	6.98
propanol-GlucA (isomer 2)	---	3.47	---	3.47
desmethyl-SA	---	0.19	---	0.19
desmethyl-carboxylic acid	---	1.51	---	1.51
desmethyl-propanol	---	0.48	0.11	0.59
carboxylic acid	---	2.48	0.20	2.68
propanol	---	---	0.20	0.20

Table is continued on next page.

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Table 5.1.1- 17 continued

Report name BCS-CN88460-	Test 6 (male, bile-duct cannulation, 2 mg/kg bw)			
	Urine (0 - 24 h)	Bile (0 - 32 h)	Faeces (0 - 24 h)	Total
desmethyl-GlucA (isomer 1)	---	3.86	---	3.86
desmethyl-GlucA (isomer 2)	---	1.64	---	1.64
2-propanol	---	---	0.07	0.07
desmethyl-propanol-GlucA (isomer 2)	---	0.77	---	0.77
desmethyl	---	---	0.38	0.38
parent compound	---	---	18.24	18.24
Total identified	**5.38**	**69.01**	**19.53**	**93.92**
Total characterised	**0.66**	**7.57**	**0.12**	**7.85**
Number of characterised metabolites (maximum value)	1 (0.16)	19 (1.36)	1 (0.12)	20 (1.46)
Solids of faeces (PES)	---	---	0.59	0.59
Urine or faeces (24 - 48 h), bile (32 - 48 h) not analysed	0.35	1.03	0.43	1.81
Fractions not analysed	---	---	0.01	0.01
Total				**104.16**
Identification rate of BCS-CN88460-carboxylic acid and its metabolites	**5.28**	**9.67**	-	**14.95**

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Table 5.1.1- 18: Test 7: Balance of BCS-CN88460 and metabolites excreted after oral administration of 2 mg/kg bw to bile-duct cannulated female rats expressed as % dose administered

Report name BCS-CN88460-	Test 7 (female, bile-duct cannulation, 2 mg/kg bw)			
	Urine (0 - 48 h)	Bile (0 - 48 h)	Faeces (0 - 48 h)	Total
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)	0.25	---	---	0.25
desfluoro-N-methyl-pyrazole-carboxylic acid	0.10	---	---	0.10
pyrazole-carboxylic acid-Ala	0.16	---	---	0.16
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys and desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys-Gly	---	0.24	---	0.24
N-methyl-pyrazole-carboxylic acid	0.13	---	---	0.13
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH	---	0.04	---	0.04
cyclopropyl-pyrazole-carboxamide	0.13	---	---	0.13
desmethyl-hydroxymethyl-diOH	---	0.66	---	0.66
desmethyl-diOH-GlucA (isomer 3)	---	1.42	---	1.42
desmethyl-diOH-GlucA (isomer 4)	---	2.71	---	2.71
desmethyl-diOH-GlucA (isomer 5)	---	2.65	---	2.65
desmethyl-OH-GlucA (isomer 1)	---	0.20	---	0.20
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	---	2.13	---	2.13
desmethyl-OH-GlucA (isomer 2)	0.11	3.45	0.10	3.66
desmethyl-hydroxyphenyl-2-propanol	0.27	1.42	---	1.69
desmethyl-lactic acid	---	4.36	---	4.36
desmethyl-hydroxymethyl-carboxylic acid	0.14	---	0.07	0.21
desmethyl-oxo-GlucA carboxylic acid-GlucA* and desmethyl-carboxylic acid-GlucA (isomer 2)*	---	4.58	---	4.58
desmethyl-propanol-GlucA (isomer 1), olefine, oxo-GlucA, lactic acid* and desmethyl-diOH-GlucA (isomer 6)	---	11.94	---	11.94
propanol-GlucA (isomer 1)	---	12.27	---	12.27
propanol-GlucA (isomer 2)	---	3.46	---	3.46
desmethyl-SA	---	0.08	---	0.08
desmethyl-carboxylic acid	0.87	2.07	---	2.94
desmethyl-propanol	---	1.15	0.08	1.23
carboxylic acid	0.13	1.35	0.06	1.54
propanol	---	---	0.05	0.05
desmethyl-GlucA (isomer 1)	---	14.09	---	14.09
desmethyl-GlucA (isomer 2)	---	7.26	---	7.26
2-propanol	---	---	0.04	0.04
desmethyl-propanol-GlucA (isomer 2)	---	4.55	---	4.55
desmethyl	---	---	0.46	0.46
parent compound	---	1.24	14.31	15.56
Total identified	**2.30**	**83.33**	**15.17**	**100.79**
Total characterised	**0.07**	**2.12**	---	**2.20**
Number of characterised metabolites (maximum value)	1 (0.07)	8 (0.63)	---	9 (0.63)
Solids of faeces (PES)	---	---	0.35	0.35
Total				**103.34**
Identification rate of BCS-CN88460-carboxylic acid and its metabolites	**1.67**	**7.78**	---	**9.46**

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Label specific metabolites

Cleavage of the phenyl moiety led to pyrazole label specific metabolites, which were not detected in the phenyl label study ([redacted], R.; [redacted], N.; 2017; M-602883-02-1). The following label specific metabolites occurred and amounted to levels up to 4.92% of the dose administered: BCS-CN88460-pyrazole-amide, BCS-CN88460-cyclopropyl-pyrazole-carboxamide-OH-GlucA, BCS-CN88460-pyrazole-carboxylic acid, BCS-CN88460-cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2), BCS-CN88460-desfluoro-N-methyl-pyrazole-carboxylic acid, BCS-CN88460-pyrazole-carboxylic acid-Ala, BCS-CN88460-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys, BCS-CN88460-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys-Gly, BCS-CN88460-N-methyl-pyrazole-carboxylic acid, BCS-CN88460-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH, BCS-CN88460-cyclopropyl-pyrazole-carboxamide and BCS-CN88460-cyclopropyl-oxy-pyrazole-carboxamide.

Metabolic pathway

In general, the main metabolic routes observed for rats treated with [pyrazole-4-^{14}C]BCS-CN88460 and [phenyl-UL-^{14}C]BCS-CN88460 were identical. Pyrazole label specific metabolites were identified.

A high number of metabolites was identified and characterised in the excreta and bile of male and female rats, suggestive for an intensive metabolism of BCS-CN88460 in the rat. The most important metabolic reactions of [pyrazole-4-^{14}C]BCS-CN88460 were the demethylation of the pyrazole moiety and the hydroxylation leading to mono-, di or tri-hydroxy compounds followed by conjugation with amino acids, glucuronic acid, and sulphuric acid.

Demethylation was one of the most prominent metabolic reactions after dosing of [pyrazole-4-^{14}C]BCS-CN88460 to male and female rats. More than 50% of the test compound was demethylated. Subsequent metabolism of desmethyl compounds was faster in the male rat compared to the female rat, indicated by low amounts of BCS-CN88460-desmethyl (1.44% of dose) in males in contrast to 11.43% for females.

Hydroxylation of [pyrazole-4-^{14}C]BCS-CN88460 occurred mainly at the propyl group in position 1 and position 2. In addition hydroxylation at the phenyl moiety and after defluorination of the difluoromethyl moiety was observed. Hydroxylation at other positions was also detected, but was not exactly located by structure elucidation.

Conjugation occurred in male and in female rats. While BCS-CN88460-desmethyl-OH-GlucA (isomer 2) is a dominant metabolite in male rats (10.24%), it was quantified to 0.23% in females, only. BCS-CN88460-desmethyl-diOH-GlucA (isomer 1 to 6), BCS-CN88460-desmethyl-triOH-GlucA, and BCS-CN88460-desmethyl-SA occurred in males only. BCS-CN88460-desmethyl-carboxylic acid-GlucA (isomer 1) was detected to 1.81% in male rats and to 1.16% in female rats. In bile samples N-conjugation was more pronounced in female rats. BCS-CN88460-desmethyl-GlucA (isomer 1) occurred up to 14.09% of the dose administered and BCS-CN88460-desmethyl-GlucA (isomer 2) up to 7.26%.

Another important metabolic reaction was the carboxylation of the 1-propanol group, leading to a carboxylic acid or with a hydroxy group in position 2 of the propyl group to a lactic acid group.

Cleavage was detected for the phenyl moiety and was leading to a cyclopropyl-pyrazole-carboxamide. Oxidation, hydroxylation and conjugation with glucuronic acid were observed as follow-up reactions. Cleavage of the phenyl moiety in combination with cleavage of the cyclopropyl ring was leading to a pyrazole-amide compound. Subsequent reactions were hydroxylation, defluorination, conjugation with glutathione and its degradation compounds, as well as conjugation with glucuronic acid and alanine, and oxidation in the pyrazole ring.

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III. CONCLUSIONS

The toxicokinetic and metabolic behaviour of [pyrazole-4-^{14}C]BCS-CN88460 can be characterised by the following facts:
BCS-CN88460 was rapidly absorbed and distributed. For all low dose tests (2 mg/kg) the mean maximum plasma concentration was reached at approximately 1 h after administration. Following administration of 200 mg/kg bw absorption was delayed. Plasma concentrations declined to values below 3.1% of the maximum concentration within 72 h post administration. The time course of the mean plasma levels was comparable in male and female rats.

Moderate amounts of radioactivity were detected in organs and tissues of all low and high dose tests. After administration of the low dose, amounts of radioactivity were in the same order of magnitude in organs of male and female rats. Following administration of the single high dose, female rats showed a slightly higher organ concentration compared to male rats, except of the liver. A lower organ concentration following pre-treatment with BCS-CN88460 than post administration of a single oral dose was observed.

Absorbed radioactivity was quickly and efficiently eliminated from the bodies of the rats and was nearly completed at sacrifice at 72 h after administration. At 24 h, more than 80% of the radioactivity was eliminated. From the elimination kinetics of the total radioactivity from plasma it was concluded that small amounts of residual radioactivity in organs and tissues are subject to further elimination.

The excretion was predominantly faecal. Urinary excretion up to 13.6% was detected in low dose tests. For high dose tests the urinary excretion was lower.

Absorption rates from the dose recovered were calculated and amounted to 80% for males and 84% for females.

BCS-CN88460 was intensively metabolised. A higher number of metabolites were detected in males compared to females. However, the total metabolism rate was comparable.

Parent compound was the main compound in faeces of high dose tests and tests with bile-duct cannulated rats.

Major metabolites in male and female rats were BCS-CN88460-desmethyl-carboxylic acid and BCS-CN88460-carboxylic acid, except for bile-duct cannulated rats. BCS-CN88460-desmethyl-hydroxyphenyl-1,2-propandiol, BCSCN88460-desmethyl-OH-GlucA (isomer 2) were found in low dose males. BCS-CN88460-desmethyl, BCS-CN88460-desmethyl-hydroxymethyl-carboxylic acid and BCS-CN88460-desmethyl-propanol were found in low dose female rats. Beside these identified metabolites a high number of prominent and minor metabolites were identified. More metabolites may be present as indicated by broad non-resolved zones in the chromatograms.

One of the most important metabolic reactions of [pyrazole-4-^{14}C]BCS-CN88460 was the demethylation of the pyrazole moiety, which occurred to more than 50%.

Other important reactions were hydroxylation in position 1 and 2 of the propyl group, in the phenyl ring, and after defluorination of the difluoromethyl moiety leading to mono-, di- or tri-hydroxy compounds. Hydroxylation in other positions was also detected, but was not exactly located by structure elucidation.

Conjugation with glucuronic acid after hydroxylation or via nitrogen was also prominent. Further observed was the conjugation with glutathione after defluorination followed by degradation of the glutathione conjugate to glycine-cysteine or cysteine conjugates. Conjugation of the BCS-CN88460-pyrazole-carboxylic acid compound with alanine and conjugation of the BCS-CN88460-desmethyl with sulphuric acid were detected.

Additional minor reactions were the oxidation of the 1-propanol group, cleavage of the phenyl moiety, cleavage of the phenyl moiety in combination with cleavage of the cyclopropyl ring, dehydration after hydroxylation of the propyl group and oxidation in the pyrazole ring.

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The results are in good agreement with the results of the ADME study with the phenyl- label ([redacted], R.; [redacted], N.; 2017; M-602883-02-1) and the pilot metabolism study ([redacted], R.; [redacted], N.; 2017; M-602456-01-1).

Based on the results, it is concluded that the toxicokinetic and the metabolic behaviour, as well as the pathway of [pyrazole-4-^{14}C]BCS-CN88460 in rats are sufficiently understood and is presented in the following figure.

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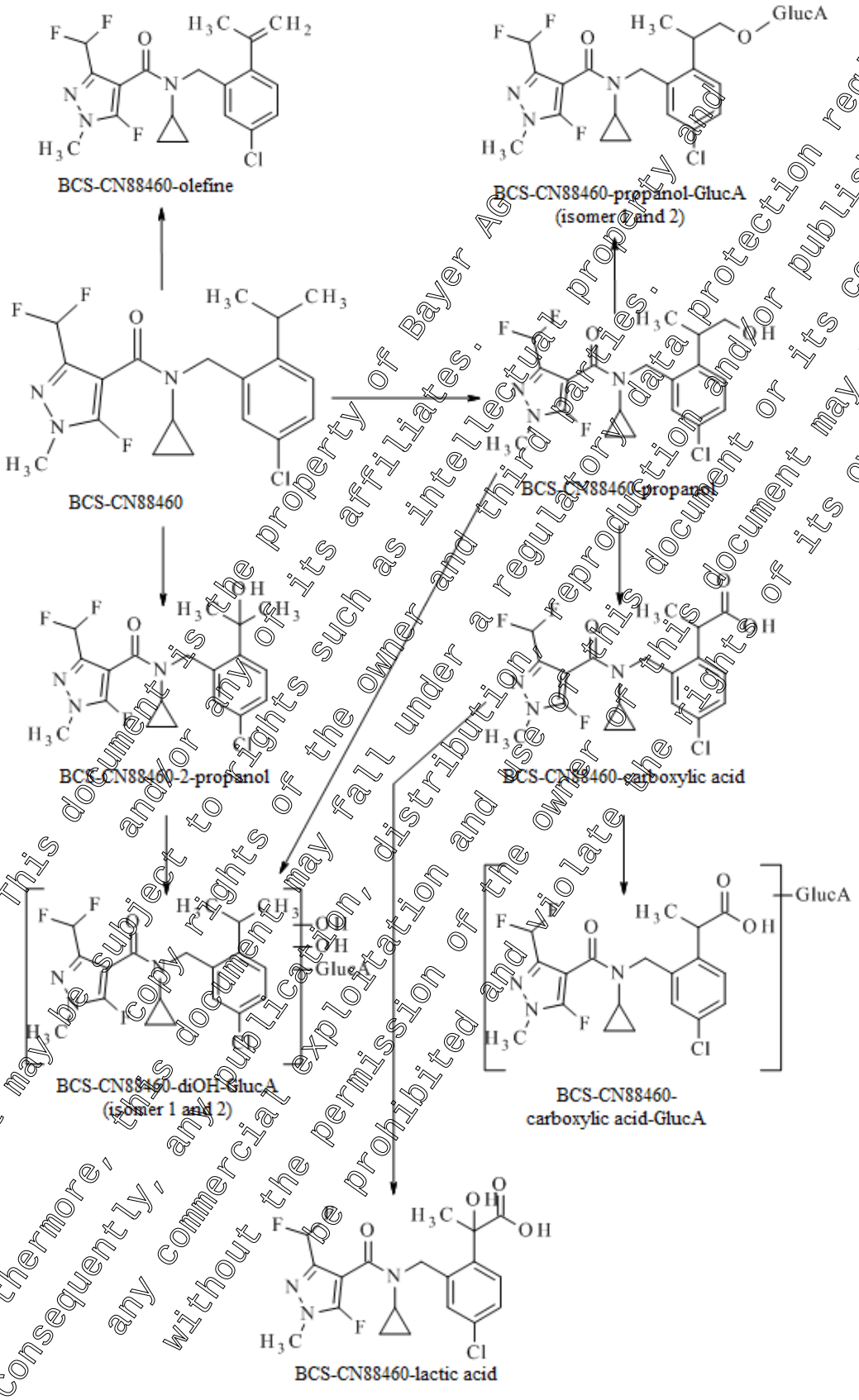
Figure 5.1.1- 1: Proposed metabolic pathway of [pyrazole-4-^{14}C]BCS-CN88460 in the rat (Part A)



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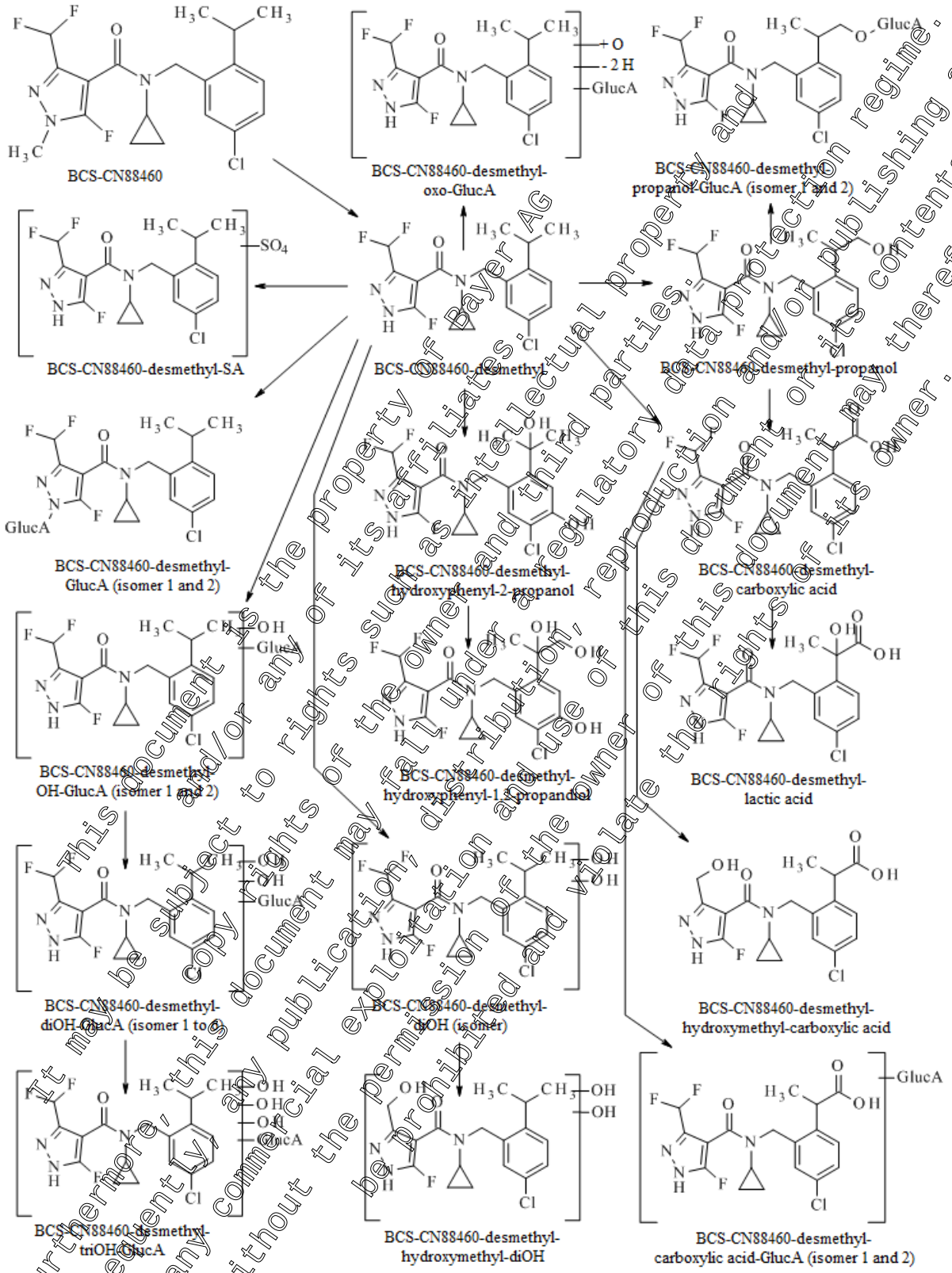
Part B of the pathway is presented on the next page

Figure 5.1.1- 1 Proposed metabolic pathway of [pyrazole-4-^{14}C]BCS-CN88460 in the rat (Part B)



Part C of the pathway is presented on the next page

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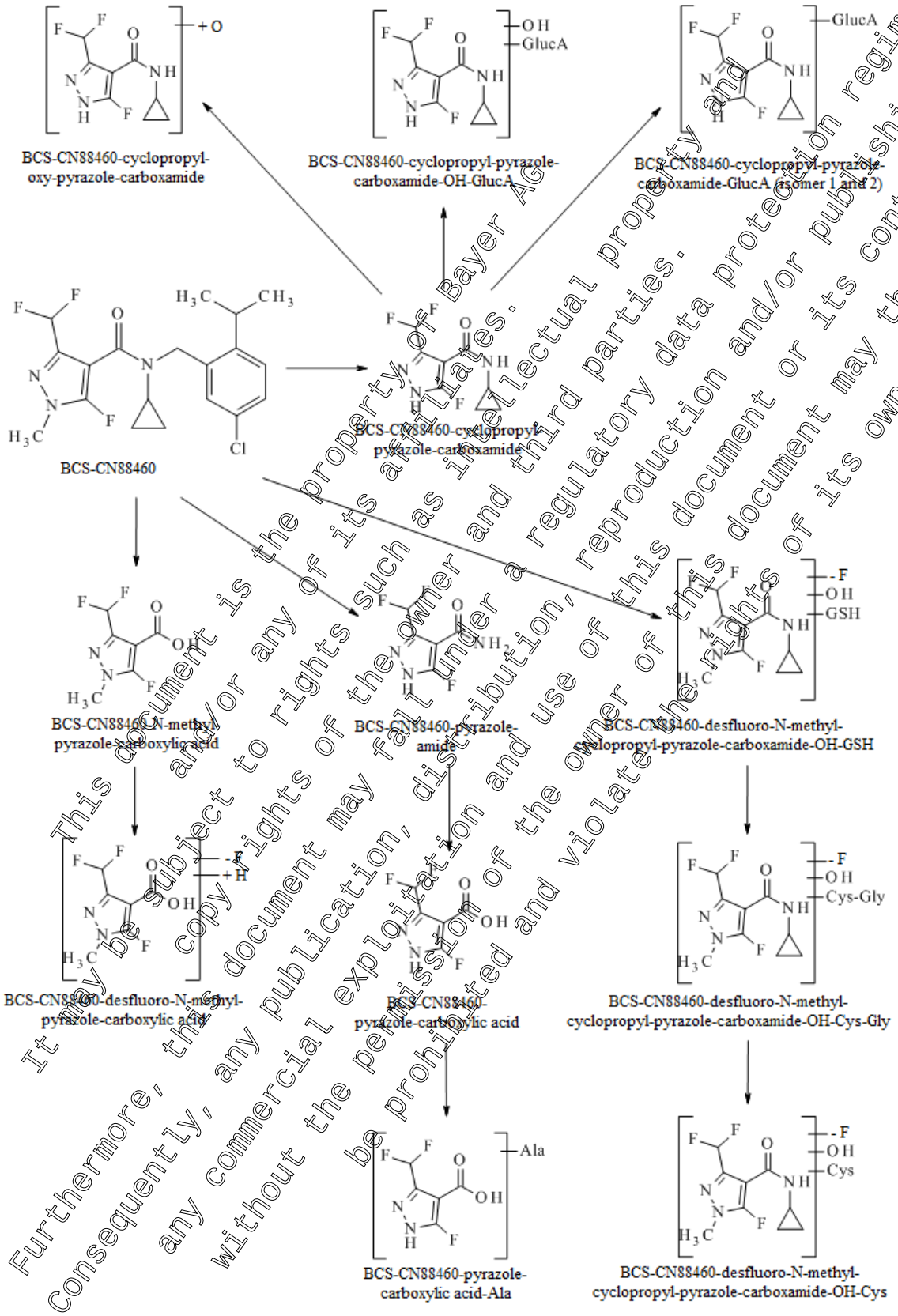
Figure 5.1.1- 1 Proposed metabolic pathway of [pyrazole-4-^{14}C]BCS-CN88460 in the rat (Part C)



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Report:	KCA 5.1.1/02; [redacted], R.; [redacted], N.; 2017; M-602883-02-1
Title:	Amendment no 1: [Phenyl-UL-14C]BCS-CN88460 - Absorption, distribution, excretion and metabolism in the rat
Report No.:	EnSa-16-1014
Document No.:	M-602883-02-1
Guideline(s):	Regulation (EC) No 1107/2009 of the European Parliament and of the Council amended by Commission Regulation (EU) No 283/2013 US EPA OCSPP 870.7485 Japanese MAFF Test Guideline 12 Nousan 8147
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

The toxicokinetic behaviour (absorption, distribution, and excretion) and metabolism of BCS-CN88460, labelled with ^{14}C in the phenyl moiety of the molecule, was investigated in male and female Wistar rats.

Two experiments with male and female rats at a low dose of 2 mg/kg bw were conducted in this study. The rats received the radioactive test compound by oral gavage as a suspension in water and Tragacanth at pH 4. They were sacrificed three days post dosing. The total radioactivity included the radioactivity related to the test compound and the metabolites and was determined in plasma, urine, faeces, organs and tissue samples at sacrifice. Metabolites were investigated in urine and extracts of faeces.

Recovery

Between 102.29% and 104.86% of the administered radioactivity were recovered by measurement of the total radioactivity in plasma samples, urine and faeces, as well as in organs and tissues at sacrifice.

Absorption

The absorption of [phenyl-UL-4-^{14}C]BCS-CN88460 started immediately after oral administration and the maximum plasma concentration (C_{max}) was reached within 1 h (t_{max}) post administration with a mean concentration of 0.417 µg/g in male rats and 0.498 µg/g in female rats. The time course of the mean plasma levels was comparable in male and female rats in all tests.

Distribution and plasma kinetics

From peak levels, the time course of radioactivity in plasma showed a decline and efficient elimination of the test substance and its metabolites from the body at the latest after 72 h post administration. The plasma concentration was calculated with a two-compartment model by TOPFIT. The weighting of $1/y^2$ was used, due to a fast elimination phase after reaching the plasma peak followed by a slower elimination phase after approximately 24 h. There were no relevant sex specific differences in the measured or calculated pharmacokinetic parameters.

Excretion

The excretion in male and female rats was almost completed 72 h after administration. At this time more than 99% of the recovered dose had been excreted via urine and faeces. In both tests the main portion of radioactivity (>80%) was excreted latest after 24 h. In both dose tests the excretion was predominantly faecal and amounted to 88.98% for males (mean value) and 91.75% of the recovered dose for females (mean value). The urinary excretion rates (mean values) were 10.66% for males and 7.99% of the recovered dose for females.

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Residues in organs and tissues at sacrifice

Generally, the amount of radioactive residues in organs and tissues of both tests was moderate. The equivalent concentration of radioactivity in organs and tissues of male and female rats was in the same order of magnitude. At sacrifice low levels of radioactivity of the administered dose (mean values: 0.290% in males and 0.234% in females) were found in the bodies excluding GIT. Low amounts of radioactivity were detected in the GITs (0.077% in males and 0.036% in females). Thus, the elimination of the test compound related radioactivity was nearly completed at sacrifice.

For all tests, the highest concentration of radioactivity in organs and tissues was detected in the liver and blood cells.

Metabolism

For investigation of the metabolism urine and faeces were sampled at various time points during both tests. Urine samples were analysed without any additional sample preparation. In all tests faeces samples were conventionally extracted with a mixture of acetonitrile/water (8/2, v/v) plus formic acid, followed by an exhaustive extraction with acetonitrile/water (1/1; v/v) and acetonitrile/water (1/1; v/v) plus formic acid using microwave assistance. The conventional extraction rates accounted for between 92.1% and 95.4% of the radioactivity. After exhaustive extraction, only up to 1.71% of the administered dose remained in the post extraction solids (PES). There were no losses during sample preparation.

Metabolites were identified in the course of the ADME study with the pyrazole label ([redacted], R.; [redacted], N.; 2017; M-602452-02-1) and assigned in the profiles of the current study by comparison of the retention times and metabolite profiles. In addition metabolites were identified in isolated fractions from selected samples of urine and conventional extract of faeces of the current study by structure elucidation.

A high number of metabolites was identified and characterised in the excreta of male and female rats, suggestive for an intensive metabolism of BCS-CN88460 in the rat. The majority of metabolites (between 87.89% and 91.86% of the administered dose) was identified (see table below). The other metabolites were characterised by their extraction and chromatographic behaviour. Probably more isomeric forms of identified metabolites may have been formed as indicated by broad non resolved zones in the chromatograms.

The metabolite profiles were qualitatively similar after dosing of 2 mg/kg bw [phenyl-UL-^{14}C]BCS-CN88460 to male and female rats. Quantitatively, sex related differences of the metabolite profiles could be observed. However, the metabolism in general was comparable in male and female rats.

The unchanged parent compound was excreted with the faeces, only and amounted to 1.08% of dose for males and 4.12% of dose for females. The most important metabolic reactions of [phenyl-UL-^{14}C] BCS-CN88460 were the demethylation of the pyrazole moiety and the hydroxylation leading to mono-, di or-tri-hydroxy compounds followed by conjugation with glucuronic acid. Another important metabolic reaction was the carboxylation of the 1-propanol group, leading to a carboxylic acid or with a hydroxy group in position 2 of the propyl group to a lactic acid group.

Major metabolites in male rats were BCS-CN88460-desmethyl-hydroxyphenyl-1,2-propandiol, BCS-CN88460-desmethyl-diOH (group of isomers), and BCS-CN88460-carboxylic acid. Major metabolites in females were BCS-CN88460-desmethyl-diOH (isomer), BCS-CN88460-desmethyl-carboxylic acid, BCS-CN88460-desmethyl-propanol, BCS-CN88460-carboxylic acid, and BCS-CN88460-propanol.

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Table 5.1.1- 19: Test 1 and 2: Balance of BCS-CN88460 and metabolites excreted with urine and faeces after oral treatment to male and female rats

Report name	Test 1 male	Test 2 female
BCS-CN88460-	oral, 2 mg/kg bw	
	% of dose administered	
benzylalcohol-dioxo-GlucA (isomer 1)	0.82	0.15
benzylalcohol-dioxo-GlucA (isomer 2)	1.99	0.54
desmethyl-triOH-GlucA	0.84	---
benzylalcohol-dioxo (isomer 1)	0.38	0.21
desmethyl-hydroxymethyl-diOH	3.31	2
benzylalcohol-dioxo (isomer 2)	0.53	0.38
desmethyl-hydroxyphenyl-1,2-propandiol	11.41	---
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	4.38	2.50
phenyl-formyl-olefine, benzylalcohol-GlucA and benzylalcohol-oxo-GlucA (isomer 1)	1.45	1.45
desmethyl-diOH (group of isomers)	14.60	1.13
benzylalcohol-oxo-GlucA (isomer 2)	1.64	0.78
desmethyl-lactic acid	6.53	4.06
desmethyl-diOH (isomer)	7.41	19.35
lactic acid	3.58	0.28
propanol-GlucA (isomer 1 and 2)	1.10	1.13
benzylalcohol-oxo-desdihydro (isomer 1)	0.14	0.11
benzylalcohol-oxo desdihydro (isomer 2)	0.11	0.26
desmethyl-SA	0.41	1.40
desmethyl-carboxylic acid	7.48	12.40
desmethyl-propanol	2.63	14.24
carboxylic acid	9.76	14.55
propanol	4.04	8.92
benzylalcohol-oxo	1.13	0.45
2-propanol	1.12	0.88
desmethyl	---	2.59
parent compound	1.08	4.12
Total identified	**87.89**	**91.86**
Total characterised	**9.35**	**7.29**
Number of characterised metabolites	17	13
Maximum value of a characterised metabolite	1.79	1.49
Exhaustive extract of faeces	2.76	3.15
Solids of faeces (PES)	1.50	1.71
Urine not analysed	0.17	0.20
Faeces not analysed	0.25	0.39
Total	**101.93**	**104.59**

In general, the main metabolic routes observed for rats treated with [phenyl-UL- ^{14}C]BCS-CN88460 and [pyrazole-4-^{14}C]BCS-CN88460 were identical. Cleavage of the pyrazole moiety led to phenyl label specific metabolites which could not be detected in the pyrazole label study ([redacted], R.; [redacted], N.; 2017; M-602452-02-1).

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The principal metabolic reactions of [phenyl-UL-^{14}C]BCS-CN88460 in the rat are listed below:

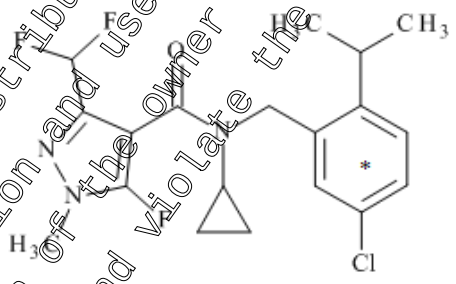
- demethylation of the pyrazole moiety
- hydroxylation in position 1 and 2 of the propyl group, in the phenyl ring and after defluorination of the difluoromethyl moiety was leading to mono-, di-, or tri-hydroxy compounds. Hydroxylation in other positions was also detected, but was not exactly located by structure elucidation
- further oxidation of the 1-propanol group was leading to a carboxylic acid group or in combination with a 2-propanol group to a lactic acid group
- cleavage of the pyrazole moiety was leading to a number of benzylalcohol compounds followed by oxidation and dehydrogenation
- conjugation with glucuronic acid after hydroxylation or via nitrogen was leading to several glucuronic acid conjugates
- conjugation of the BCS-CN88460-desmethyl with sulphuric acid
- dehydrogenation of the propyl group after cleavage of the pyrazole moiety was leading to BCS-CN88460-phenyl-formyl-olefine

Based on these results it is concluded that the bio kinetic and metabolic behaviour of [phenyl-UL-^{14}C]BCS-CN88460 in rats is sufficiently understood and a metabolic pathway is proposed.

I. Material and Methods

A. Material

1. Test material:

Test substance	
Chemical structure	 *denotes the position of the ^{14}C-radiolabel
Radiolabelled test material	[Phenyl-UL-^{14}C]BCS-CN88460
Specific radioactivity	4.13 MBq/mg (111.51 µCi/mg)
Radiochemical purity	>99% for all aliquots of the test compound certified by HPLC (radio- and UV detection)
Chemical purity	>99% (HPLC)
Vehicle	0.5% aqueous Tragacanth solution
Preparation of dosing solution	Radiolabelled test compound suspended aqueous Tragacanth solution

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2. Test Animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	Wistar Unilever (WU) breeder strain: HsdCpb:WU (males) and Crl:(Wi)WU BR (females)
Breeding facility:	Males: [redacted] Females: [redacted]
Sex and numbers involved:	Test 1: 4 male rats Test 2: 4 female rats
Age:	About 7 weeks for male rats and about 9 weeks for female rats at the time of delivery
Body weight:	Males of Test 1: 187 - 196 g at the time of administration 178 - 195 g at the time of sacrifice Females of Test 2: 196 - 202 g at the time of administration 189 - 203 g at the time of sacrifice
Acclimatization:	The animals were acclimated to the laboratory conditions in Makrolon cages on wood shavings in the test facility for about 1 week prior to the administration.
Identification:	The animals were identified by cage cards listing the study number, test compound name and individual animal number. They were additionally labelled with water-insoluble spots on the tail.
Housing:	The animals were kept under conventional hygienic conditions in air-conditioned rooms. After administration of the radiolabelled test compound, the animals were kept individually in Makrolon metabolism cages. With these cages, an almost quantitative and separate collection of urine and faeces was possible. Temperature 20 - 23 °C, relative humidity 19 - 83 %. 12 / 12 hours light / dark cycle.
Feed and water:	The rats were fed *ad libitum* with rat/mice maintenance long life diet (V1574-000 Ered LR/M-H, 10 mm, supplied by [redacted]). They received approximately 16 g per animal and day. Tap water from municipal water supply, *ad libitum*

B. Study design

1. Dosing

For each administration suspension, an adequate aliquot of the corresponding stock solution was concentrated to near dryness under a gentle stream of nitrogen.

For each test, the individual portions of test compound were formulated with 0.5% aqueous Tragacanth using an ultrasonic bath. Afterwards the individual administration suspensions were stirred on a magnetic stirrer overnight at approx. 5°C and at room temperature during the administration process.

After each radioactive dosing of rats, the identity and the radiochemical purity of the test compound in the administration suspension was confirmed by HPLC analysis

Rats were orally administered by gavage using a syringe attached to an animal-feeding knob cannula.

The concentration of each administration suspension was calculated to achieve an administered amount of about 2 mg test compound per kg body weight (bw). After administration of the radiolabelled test compound the rats were kept individually in Makrolon metabolism cages, which allowed separate and quantitative collection of urine and faeces. Plasma, urine and faeces samples

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were collected at various times separately for each animal. Organs and tissues were dissected from the animals at sacrifice. The collection intervals for the respective samples are summarized in the table below.

Table 5.1.1- 20: Experiments conducted in the ADME study with [phenyl-UL-^{14}C]BCS-CN88460

Test no.	Test description / Date of dosing / Date of sacrifice	Time of sample collection			
		Urine [h]	Faeces [h]	Organs [h]	Microplasma [h]
1	4 male rats single oral dose (2 mg/kg bw)	4, 8, 12, 24, 48, 72	24, 48, 72	72	0.25, 0.5, 1, 2, 4, 7, 24, 48, 72
2	4 female rats single oral dose (2 mg/kg bw)	4, 8, 24, 48, 72	24, 48, 72	72	

2. Sample collection

Collection of plasma

Blood samples were collected separately as micro sample for each animal by pressing a capillary coated with heparin in a small cut in the tail vein. The wound was closed with adhesive tape. The capillaries were centrifuged at approximately 12000 g for 10 minutes using a haematocrit centrifuge to separate plasma from the blood cells. By this method, it was possible to collect plasma samples at different time points from the same animals, and plasma curves from single animals were generated. This procedure results in lower variability compared to curves calculated from whole blood samples of different animals by avoiding inter-animal variation.

Collection of excreta

Immediately after the administration of the test compound, animals were transferred to metabolism cages. Urine and faeces were collected separately and quantitatively for each animal. Urine was collected in a cryogenic trap with dry ice. The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction and radioactivity was determined by LSC (liquid scintillation counting).

Collected faeces were weighed, diluted with water at a ratio of about 1:1 (w/v), homogenised before aliquots were taken for the determination of radioactivity combustion/LSC.

Sacrifice and organ/tissue sampling

All animals were sacrificed in anaesthesia by transection of the cervical blood vessels at 72 hrs after dosing to obtain blood, organs and tissues. The blood collected at sacrifice was separated into plasma and erythrocytes by centrifugation. The organs and tissues were dissected, weighed immediately and lyophilised. Finally, they were homogenised before aliquots were taken for the determination of radioactivity by combustion/LSC. For small organs and tissues such as adrenal glands, thyroid, ovaries, renal fat and uterus, only the wet weight was determined before the organs were solubilised followed by radio assay by LSC. The radioactivity remaining in the GIT, skin and residual carcass was determined by combustion/LSC in order to establish the radioactivity balance.

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3. Measurement of radioactivity

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

4. Toxicokinetic analysis

In this study, the software TOPFIT version 2.0 was used to calculate toxicokinetic parameters by plasma concentration-time curve analysis for the mean dose normalised equivalent concentration-values from each test. A standard 2-compartment disposition model was applied for curve fitting computation. Compartments are defined as physical locations in the body that can be represented with certain simplifications during modelling. Compartment models attempt to describe the following processes mathematically: absorption of the administered compound, entry into the systemic circulation, distribution into organs or tissues where metabolism can occur and subsequent excretion.

A list of the toxicokinetic parameters that is addressed in this study is given in the table below.

$AUC_{0-\infty}$	Total area under the plasma radioactivity concentration-time curve extrapolated from time 0 to infinity [mg/kg x h for "C", g/g x h for "C_{norm}"]
$t_{1/2\ e1}$	Half-life of the distribution and elimination phase [h]
$t_{1/2\ e2}$	Half-life of the terminal elimination phase [h]
t_{max}	Time at which the maximum radioactivity concentration occurs in plasma following administration of an extravascular dose [h]
C_{max}	Maximum radioactivity concentration observed in plasma following administration of an extravascular dose [mg/kg for "C", g/g for "C_{norm}"]

5. Metabolite analysis

For analysis and quantification of the parent compound and metabolites in urine and faeces, respective samples from all animals of a test were pooled, and samples from these pools were used for characterisation, identification and quantification.

Urine samples were analysed directly, without a preceding sample preparation by HPLC based on reversed phase chromatography using an acidic water/acetonitrile/tetrahydrofurane gradient. Pooled faeces samples were conventionally and exhaustively extracted with mixtures of acetonitrile, water and formic acid. Prominent metabolites were identified in isolated fractions from the urine (test 1, 8 - 12 h) and in isolated fractions from the conventional extract of feaces of test 2 (0 - 48 h). The fractions were purified using different column types and solvent gradients. The metabolites in the fractions were identified based on spectroscopic methods or assignment of the retention times.

Identical metabolites in urine and extract of faeces of the current study were assigned to each other based on their retention times and their spectroscopic behaviour and were named with the same report name. Identical identified metabolites in the current study and the ADME study with the pyrazole label were also named with the same report name. The assignment of these metabolites in the profiles was achieved by comparison of the retention times and metabolite profiles.

An overview on the reference compounds is given in Table 5.1.1- 21.

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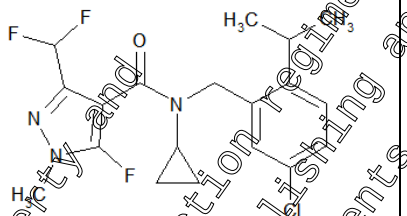
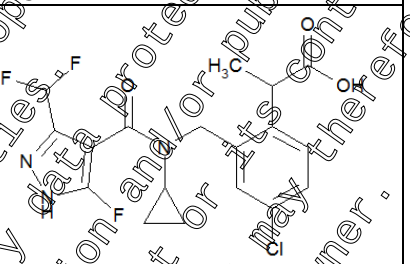
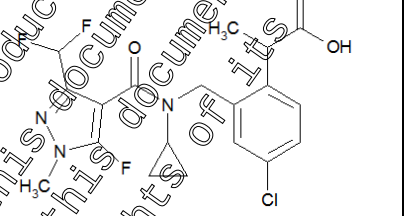
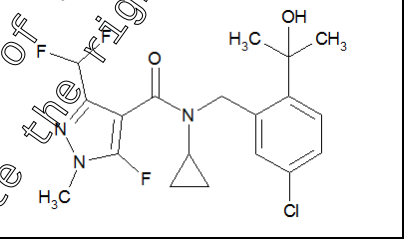
Table 5.1.1- 21: List of reference compounds

Report name/ BCS codes	**Chemical name (IUPAC)**	**Chemical Structure**
Parent compound **BCS-CN88460**	N-(5-chloro-2-isopropylbenzyl)-N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazole-4-carboxamide	
BCS-CN88460-desmethyl-carboxylic acid BCS-CX99799 sample ID: HF76B45	2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1H-pyrazol-4-yl]carbonyl}amino)methyl]phenyl}propanoic acid	
BCS-CN88460-carboxylic acid BCS-CY26497 sample ID: HF76B47	2-[4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazol-4-yl]carbonyl}amino)methyl]phenyl}propanoic acid	
BCS-CN88460-2-propanol BCS-DC20298 sample ID: YK0614H1	N-[5-chloro-2-(2-hydroxypropan-2-yl)benzyl]-N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazole-4-carboxamide	

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II. RESULTS AND DISCUSSION

A. Recovery

Between 102.29% and 104.86% of the administered radioactivity were recovered by measurement of the total radioactivity in plasma samples, urines and faeces, as well as in organs and tissues at sacrifice. Details on the recovery of radioactivity administered to male and female rats are summarised in the following tables.

Table 5.1.1- 22: Recovery of radioactivity in excreta, gastrointestinal tract and the body of rats following oral dosing of [phenyl-UL-^{14}C]BCS-CN88460

	Percent of total radioactive dose administered (mean values)		Percent of total radioactive dose recovered (mean values)	
	Test 1 male oral 2 mg/kg bw	**Test 2** female oral 2 mg/kg bw	**Test 1** male oral 2 mg/kg bw	**Test 2** female oral 2 mg/kg bw
Urine	10.89	8.39	10.66	7.99
Faeces	91.04	96.21	88.98	91.75
Total excreted	**101.93**	**104.59**	**99.64**	**99.74**
Body excluding GIT	0.290	0.234	0.283	0.223
GIT	0.077	0.036	0.076	0.034
Total in body	**0.367**	**0.270**	**0.359**	**0.257**
Balance	**102.29**	**104.86**	**---**	**---**
Norm. - factor	-	-	0.978	0.954

B. Absorption

The absorption of [phenyl-UL-^{14}C]BCS-CN88460 started immediately after oral administration as shown by the concentration profile of the radioactivity in the plasma at individual time points. For both tests the dose normalized mean maximum plasma concentration (C_{max}) was reached at 1 h (t_{max}) after administration with a mean concentration of 0.417 µg/g in male rats and 0.498 µg/g in female rats, respectively.

The time course of the mean plasma levels was comparable in male and female rats.

Radioactivity (below 1.93% of the maximum plasma concentration measured) could be detected in all plasma samples until 72 h after dosing, the latest time of plasma sampling.

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Table 5.1.1- 23: Time course of radioactivity in the plasma of male and female rats following an oral dose of [phenyl-UL-^{14}C]BCS-CN88460

	Equivalent concentration [mg active substance equivalent / kg sample] (mean values)		Dose normalised concentration C/D (mean values)	
Time [h post admin.]	Test 1 oral male 2 mg/kg bw	Test 2 oral female 2 mg/kg bw	Test 1 oral male 2 mg/kg bw	Test 2 oral female 2 mg/kg bw
0.25	0.1995	0.1626	0.1222	0.0864
0.5	0.3849	0.3739	0.2356	0.1990
1	0.4166	0.4975	0.2551	0.2648
2	0.3334	0.4344	0.2043	0.2315
4	0.2347	0.2513	0.1440	0.1337
7	0.1610	0.1913	0.0988	0.1017
24	0.0238	0.0531	0.0146	0.0283
48	0.0109	0.0151	0.0067	0.0081
72	0.0075	0.0096	0.0046	0.0051

C. Distribution and plasma kinetics

After a single oral administration of 2 mg/kg bw [phenyl-UL-^{14}C]BCS-CN88460 to male and female rats, the dose normalised mean maximum plasma concentration of the radioactivity was measured at 1 h post administration and amounted to 0.255 for males and 0.265 for females.

For both tests the mean concentration of the total radioactivity in plasma declined to values below 1.93% of the maximum concentration within 72 h post administration. This indicates no retention of compound related residues in the body of the animals.

The plasma concentration was calculated with a two-compartment model by TOPFIT. The weighting of $1/y^2$ was used, due to a fast elimination phase after reaching the plasma peak followed by a slower elimination phase after approximately 24 h.

There were no relevant sex specific differences in the measured or calculated pharmacokinetic parameters.

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Table 5.1.1- 24: Pharmacokinetic parameters of [phenyl-UL-^{14}C]BCS-CN88460 after oral administration to male and female rats, derived from plasma curve analysis

		Test 1	Test 2
		male	female
Nominal dose (mg/kg bw)		2	2
Kind of administration		oral	oral
Actual dose (mg/kg bw)		1.63	1.88
Compartment model		two	two
Weighting of the TOPFIT calculation		$1/y^2$	$1/y^2$
t_{max}	[h] measured	1	1
t_{max}	[h] calculated	0.72	0.83
C_{max}	[µg/mL] measured	0.255	0.265
C_{max}	[µg/mL] calculated	0.248	0.222
$t_{1/2\ a}$	[h]	0.10	0.11
$t_{1/2\ e}$	[h]	44.5	31.1
$AUC_{0-\infty}$	[g/g*h]	2.43	2.75

D. Excretion

The excretion in male and female rats was almost completed 72 h after administration. At this time more than 99% of the recovered dose had been excreted via urine and faeces. In both tests the main portion of radioactivity (~ 73%) was excreted latest after 24 h.

In both dose tests the excretion was predominantly faecal and amounted to 88.98% for males (mean value) and 91.75% of the recovered dose for females (mean value). The mean urinary excretion of total radioactivity after 72 h was higher in male rats (10.66%) than in female rats (7.99% of the recovered dose). The urinary excretion was slightly faster in male rats than in female rats.

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Table 5.1.1- 25: Cumulative excretion of radioactivity at time intervals

Time [h post admin.]	Percent of total radioactive dose administered (mean values)		Percent of total radioactive dose recovered (mean values)	
	Test 1 male oral 2 mg/kg bw	**Test 2** female oral 2 mg/kg bw	**Test 1** male oral 2 mg/kg bw	**Test 2** female oral 2 mg/kg bw
Urine				
4	2.47	1.23	2.41	1.18
8	3.84	3.18	3.73	3.03
12	8.11	---	7.94	---
24	10.10	7.23	9.89	6.89
48	10.72	8.19	10.49	7.81
72	10.89	8.39	10.66	7.99
Faeces				
24	87.48	76.98	85.49	73.45
48	90.79	95.82	88.74	91.38
72	91.04	96.21	88.98	91.75
Sum excreted:	**101.93**	**104.59**	**99.64**	**99.74**
Norm.-factor	---	---	0.978	0.954

E. Residues in organs and tissues at sacrifice

Generally, the amount of radioactive residues in organs and tissues of both tests was moderate. The equivalent concentration of radioactivity in organs and tissues of male and female rats was in the same order of magnitude. At sacrifice low levels of radioactivity of the administered dose (mean values: 0.290% in males and 0.234% in females) were found in the bodies excluding GIT. Low amounts of radioactivity were detected in the GITs (0.077% in males and 0.036% in females). Thus, the elimination of the test compound related radioactivity was nearly completed at sacrifice.

The highest concentration of radioactivity was detected in the liver with mean equivalent concentrations of 0.0347 mg/kg in males and 0.0374 mg/kg in female rats. The concentration in blood cells was high compared to the concentrations in organs and tissues excluding liver. Mean equivalent concentrations were 0.0225 mg/kg in blood cells of male rats and 0.0295 mg/kg in blood cells of female rats. The mean equivalent concentration in the other organs and tissues ranged from 0.0011 mg/kg to 0.0115 mg/kg.

From the elimination kinetics of the total radioactivity from plasma it can be concluded that small amounts of residual radioactivity in organs and tissues are subject to further elimination.

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Table 5.1.1- 26: Radioactive residues in organs and tissues at sacrifice after oral administration of [phenyl-UL-^{14}C]BCS-CN88460

	Equivalent concentration [mg active substance equivalent / kg sample] (mean values)		Dose normalised concentration (mean values)	
Organs/ Tissues	Test 1 oral male 2 mg/kg bw	Test 2 oral female 2 mg/kg bw	Test 1 oral male 2 mg/kg bw	Test 2 oral female 2 mg/kg bw
Blood Cells	0.0225	0.0295	0.0134	0.0156
Plasma	0.0074	0.0071	0.0044	0.0038
Carcass	0.0035	0.0029	0.0021	0.0015
Heart	0.0069	0.0057	0.0041	0.0030
Brain	0.0016	0.0011	0.0010	0.0006
Kidneys	0.0115	0.0090	0.0069	0.0047
Liver	0.0347	0.0374	0.0207	0.0197
Testes	0.0019	---	0.0011	---
Ovaries	---	0.0046	---	0.0025
Uterus	---	0.0049	---	0.0026
Adrenal gland	0.0078	0.0057	0.0047	0.0030
Harderian gland	0.0048	0.0039	0.0029	0.0021
Thyroid gland	n.c.	n.c.	n.c.	n.c.
Spleen	0.0053	0.0099	0.0032	0.0052
Lung	0.0083	0.0096	0.0050	0.0050
Eyes	0.0014	0.0013	0.0009	0.0007
Skin	0.0033	0.0038	0.0019	0.0020
Bone femur	0.0024	n.c.	0.0014	n.c.
Perirenal fat	0.0113	0.0101	0.0068	0.0053
Muscle leg	0.0021	0.0017	0.0013	0.0009

n.c. not calculated
--- no sample collected

F. Metabolism

For investigation of the metabolism urine and faeces were sampled at various time points during both tests.

Urine samples were analysed without any additional sample preparation. In all tests faeces samples were conventionally extracted with a mixture of acetonitrile/water (8/2, v/v) plus formic acid, followed by an exhaustive extraction with acetonitrile/water (1/1; v/v) and acetonitrile/water (1/1; v/v) plus formic acid using microwave assistance. The conventional extraction rates amounted to 95.4% for faeces from test 1 (0 - 24 h), 92.1% from test 1 (24 – 48 h) and 94.9% of the radioactivity from test 2 (0 – 48 h). After exhaustive extraction 1.24% of the administered dose was detected in the post extraction solids from test 1 (0 - 24 h) and 1.71% from test 2 (0 - 48 h), only. There were no losses during sample preparation.

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Parent compound and metabolites were analysed and quantified in urine samples and in the extracts of faeces by radio-HPLC. The metabolite pattern in the urine samples and extracts of faeces from the current study and the ADME study with the pyrazole label ([redacted], R.; [redacted], N.; 2017; M-602452-02-1) was similar, except of the individual label specific metabolites. Corresponding metabolites in urine and faeces extracts from all ADME rat studies with BCS-CN88460 were assigned, depending on their retention times based on the HPLC profiling method.

Metabolites were identified in the course of the ADME study with the pyrazole label ([redacted], R. [redacted], N.; 2017; M-602452-02-1) and assigned in the profiles of the current study by comparison of the retention times and metabolite profiles. In addition metabolites were identified in isolated fractions from urine (test 1, male, 8 - 12 h) and conventional extract of faeces (test 1, male, 0 - 24 h) of the current study by structure elucidation.

The metabolite profiles were qualitatively similar after dosing of 2 mg/kg bw [phenyl-UL-^{14}C]BCS-CN88460 to male and female rats. Quantitatively, sex related differences of the metabolite profiles could be observed. However, the metabolism in general was comparable in male and female rats.

The identification rates of parent compound and metabolites were high and amounted to between 87.89% for males and 91.86% of the administered dose for females.

Parent compound and metabolites

Parent compound was excreted with the faeces, only and amounted to 1.08% of dose for males and 4.12% of dose for females.

Major metabolites in male rats were BCS-CN88460-desmethyl-hydroxyphenyl-1,2-propandiol (11.41% of dose), BCS-CN88460-desmethyl-diOH (group of isomers, 14.60% of dose), and BCS-CN88460-carboxylic acid (9.76% of dose). Major metabolites in females were BCS-CN88460-desmethyl-diOH (isomer, 19.35% of dose), BCS-CN88460-desmethyl-carboxylic acid (12.40% of dose), BCS-CN88460-desmethyl-propanol (14.24% of dose), BCS-CN88460-carboxylic acid (14.55% of dose), and BCS-CN88460-propanol (8.92% of dose).

Prominent metabolites for males were BCS-CN88460-desmethyl-hydroxymethyl-diOH, BCS-CN88460-desmethyl-diOH (isomer), BCS-CN88460-lactic acid, BCS-CN88460-desmethyl-carboxylic acid, BCS-CN88460-desmethyl-propanol, and BCS-CN88460-propanol. BCS-CN88460-desmethyl was found in female rats. BCS-CN88460-desmethyl-carboxylic acid-GlucA (isomer 1), and BCS-CN88460-diOH-GlucA (isomer 1 and 2) in one region and BCS-CN88460-desmethyl-lactic acid were detected as prominent in both genders. All these metabolites amounted to above 2.50% of dose, but not more than 7.48% of dose.

A high number of metabolites amounted to < 2.0% of dose and were identified as BCS-CN88460-benzylalcohol-dioxo-GlucA (isomer 1) and (isomer 2), BCS-CN88460-desmethyl-triOH-GlucA, BCS-CN88460-benzylalcohol-dioxo (isomer 1 and 2), BCS-CN88460-phenyl-formyl-olefine, BCS-CN88460-benzylalcohol-GlucA, BCS-CN88460-benzylalcohol-oxo-GlucA (isomer 1 and 2), BCS-CN88460-desmethyl-diOH (group of isomers), BCS-CN88460-propanol-GlucA (isomer 1 and 2), BCS-CN88460-benzylalcohol-oxo-desdihydro (isomer 1 and 2), BCS-CN88460-desmethyl-SA, BCS-CN88460-benzylalcohol-oxo, and BCS-CN88460-2-propanol.

More metabolites may be present as indicated by broad non-resolved zones in the chromatograms. Unidentified metabolites were characterised by their extraction and chromatographic behaviour.

All results correspond well with the findings in the ADME study with the pyrazole label.

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Table 5.1.1- 27: Test 1: Balance of BCS-CN88460 and metabolites excreted after oral administration of 2 mg/kg bw to male rats

Report name BCS-CN88460-	Test 1 (male, 2 mg/kg bw)		
	Urine (0 – 48 h)	Faeces (0 – 48 h)	Total
	% of dose administered		
benzylalcohol-dioxo-GlucA (isomer 1)	0.82	---	0.82
benzylalcohol-dioxo-GlucA (isomer 2)	1.99	---	1.99
desmethyl-triOH-GlucA	---	0.84	0.84
benzylalcohol-dioxo (isomer 1)	0.38	---	0.38
desmethyl-hydroxymethyl-diOH	---	3.31	3.31
benzylalcohol-dioxo (isomer 2)	0.55	---	0.55
desmethyl-hydroxyphenyl-1,2-propandiol	---	11.41	11.41
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	---	4.38	4.38
phenyl-formyl-olefine, benzylalcohol-GlucA and benzylalcohol-oxo-GlucA (isomer 1)	1.45	---	1.45
desmethyl-diOH (group of isomers)	---	14.60	14.50
benzylalcohol-oxo-GlucA (isomer 2)	1.64	---	1.64
desmethyl-lactic acid	---	6.53	6.53
desmethyl-diOH (isomer)	---	7.41	7.41
lactic acid	---	3.58	3.58
propanol-GlucA (isomer 1 and 2)	---	1.10	1.10
benzylalcohol-oxo-desdihydro (isomer 1)	0.14	---	0.14
benzylalcohol-oxo desdihydro (isomer 2)	0.11	---	0.11
desmethyl-SA	---	0.41	0.41
desmethyl-carboxylic acid	---	7.48	7.48
desmethyl-propanol	---	2.63	2.63
carboxylic acid	---	9.76	9.76
propanol	---	4.04	4.04
benzylalcohol-oxo	1.13	---	1.13
2-propanol	---	1.12	1.12
parent compound	---	1.08	1.08
Total identified	**8.21**	**79.68**	**87.89**
Total characterised	**2.51**	**6.84**	**9.35**
Number of characterised metabolites (maximum value)	11 (0.38)	6 (1.79)	17 (1.79)
Exhaustive extract of faeces	---	2.76	2.76
Solids of faeces (PES)	---	1.50	1.50
Urine or faeces not analysed (48 - 72 h)	0.17	0.25	0.42
Total			**101.93**

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Table 5.1.1- 28: Test 2: Balance of BCS-CN88460 and metabolites excreted after oral administration of 2 mg/kg bw to female rats

Report name BCS-CN88460-	Test 2 (female, 2 mg/kg bw)		
	Urine (0 – 48 h)	Faeces (0 – 48 h)	Total
	% of dose administered		
benzylalcohol-dioxo-GlucA (isomer 1)	0.15	---	0.15
benzylalcohol-dioxo-GlucA (isomer 2)	0.54	---	0.54
benzylalcohol-dioxo (isomer 1)	0.21	---	0.21
benzylalcohol-dioxo (isomer 2)	0.38	---	0.38
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	---	2.50	2.50
phenyl-formyl-olefine, benzylalcohol-GlucA and benzylalcohol-oxo-GlucA (isomer 1)	1.45	---	1.45
desmethyl-diOH (group of isomers)	---	1.13	1.13
benzylalcohol-oxo-GlucA (isomer 2)	0.78	---	0.78
desmethyl-lactic acid	---	4.06	4.06
desmethyl-diOH (isomer)	---	19.35	19.35
lactic acid	---	0.28	0.28
propanol-GlucA (isomer 1 and 2)	---	1.13	1.13
benzylalcohol-oxo-desdihydro (isomer 1)	0.11	---	0.11
benzylalcohol-oxo desdihydro (isomer 2)	0.26	---	0.26
desmethyl-SA	---	1.40	1.40
desmethyl-carboxylic acid	1.31	11.09	12.40
desmethyl-propanol	---	14.24	14.24
carboxylic acid	0.39	14.16	14.55
propanol	---	8.92	8.92
benzylalcohol-oxo	0.45	---	0.45
2-propanol	---	0.88	0.88
desmethyl	---	2.59	2.59
parent compound	---	4.12	4.12
Total identified	**6.03**	**85.83**	**91.86**
Total characterised	**2.16**	**5.13**	**7.29**
Number of characterised metabolites (maximum value)	8 (0.62)	5 (1.49)	13 (1.49)
Exhaustive extract of faeces	---	3.15	3.15
Solids of faeces (PES)	---	1.71	1.71
Urine or faeces not analysed (48 - 72 h)	0.20	0.39	0.59
Total			**104.59**

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Label specific metabolites

Cleavage of the pyrazole moiety led to phenyl label specific metabolites which could not be detected in the pyrazole label study. The following label specific metabolites occurred at levels up to 2.81% of the dose administered and were detected in urine, only: BCS-CN88460-benzylalcohol-dioxo GlucA (isomer 1 and 2), BCS-CN88460-benzylalcohol-oxo-GlucA (isomer 1 and 2), BCS-CN88460-phenyl-formyl-olefine, BCS-CN88460-benzylalcohol-GlucA, BCS-CN88460-benzylalcohol-oxo, BCS-CN88460-benzylalcohol-dioxo (isomer 1 and 2), and BCS-CN88460-benzylalcohol-oxo-desdihydro (isomer 1 and 2).

Metabolic pathway

In general, the main metabolic routes observed for rats treated with [phenyl-UL- ^{14}C]BCS-CN88460 and [pyrazole-4-^{14}C]BCS-CN88460 were identical. Phenyl label specific metabolites were identified.

A high number of metabolites was identified and characterised in the excreta of male and female rats, suggestive for an intensive metabolism of BCS-CN88460 in the rat. The most important metabolic reactions of [phenyl-UL-^{14}C]BCS-CN88460 were the demethylation of the pyrazole moiety and the hydroxylation leading to mono-, di or-tri-hydroxy compounds followed by conjugation with glucuronic acid.

Demethylation was one of the most prominent metabolic reactions. More than 50% of the test compound was demethylated. Additional metabolism of desmethyl compounds in male and female rats was observed.

Hydroxylation of [phenyl-UL-^{14}C]BCS-CN88460 occurred mainly in the propyl group in position 1 and 2. In addition hydroxylation at the phenyl moiety and after defluorination of the difluoromethyl moiety was observed. Hydroxylation in other positions was also detected, but was not exactly located by structure elucidation.

Conjugation occurred in male and in female rats. Overall, conjugation with glucuronic acid amounted to a total of approximately 11% of the administered dose and was more pronounced in male rats. In low amounts conjugation with sulphuric acid was also observed.

Another important metabolic reaction was the carboxylation of the 1-propanol group, leading to a carboxylic acid or with a hydroxy group in position 2 of the propyl group to a lactic acid group.

Cleavage of the pyrazole moiety was leading to a number of benzylalcohol compounds and BCS-CN88460-phenyl-formyl-olefine. Subsequent reactions of the benzylalcohol compounds were oxidation as well as conjugation with glucuronic acid.

III. CONCLUSIONS

The toxicokinetic and metabolic behaviour of [phenyl-UL-^{14}C]BCS-CN88460 can be characterised by the following findings:
BCS-CN88460 was rapidly absorbed and distributed. The mean maximum plasma concentration was reached at approximately 1 h after administration to male and female rats. Plasma concentrations declined to values below 1.93% of the maximum concentration within 72 h post administration. The time course of the mean plasma levels was comparable in male and female rats.

Moderate amounts of radioactivity were detected in organs and tissues. Amounts of radioactivity were in the same order of magnitude in the organs of male and female rats.

Absorbed radioactivity was quickly and efficiently eliminated from the bodies of the rats of both sexes. The elimination was nearly completed at sacrifice 72 h after administration. At 24 h the main portion of radioactivity was eliminated. From the elimination kinetics of the total radioactivity from plasma it was concluded that small amounts of residual radioactivity in organs and tissues are subject to further elimination.

The excretion was predominantly faecal. Urinary excretion up to 10.66% was observed.

Qualitatively and quantitatively sex related differences of the individual metabolites could be observed. However, the metabolism was comparable in male and female rats.

Only minor amounts of parent compound were excreted with the faeces.

Major metabolites in male rats were BCS-CN88460-desmethyl-hydroxyphenyl-1,2-propandiol, BCS-CN88460-desmethyl-diOH (group of isomers), and BCS-CN88460-carboxylic acid, while major metabolites in females were BCS-CN88460-desmethyl-diOH (isomer), BCS-CN88460-desmethyl-carboxylic acid, BCS-CN88460-desmethyl-propanol, BCS-CN88460-carboxylic acid, and BCS-

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CN88460-propanol. Beside these identified metabolites a high number of prominent and minor metabolites were identified. More metabolites may be present as indicated by broad non-resolved zones in the chromatograms.

One of the most important metabolic reactions of [phenyl-UL-^{14}C]BCS-CN88460 was the demethylation of the pyrazole moiety, which occurred to more than 50% of dose administered. Other important reactions were hydroxylation in position 1 and 2 of the propyl group, in the phenyl ring, and after defluorination of the difluoromethyl moiety, leading to mono-, di-, or tri-hydroxy compounds. Hydroxylation in other positions was also detected, but was not exactly located by structure elucidation. Conjugation of hydroxy compounds with glucuronic acid was also prominent. Conjugation with sulphuric acid was observed. Cleavage of the pyrazole moiety was leading to a number of benzylalcohol compounds and BCS-CN88460-phenyl-formyl-olefine. Additional reactions were the oxidation of the 1-propanol group and non-specific oxidation.

The results are in good agreement with the results of the ADME study with the pyrazole label ([redacted], R.; [redacted], N.; 2017; M-602452-02-1) and the pilot metabolism study ([redacted], R.; [redacted], N.; 2017; M-602456-01-1).

Based on the results, it is believed that the toxicokinetic and the metabolic behaviour of [phenyl-UL-^{14}C]BCS-CN88460 in rats are sufficiently understood and is presented in the following figure.

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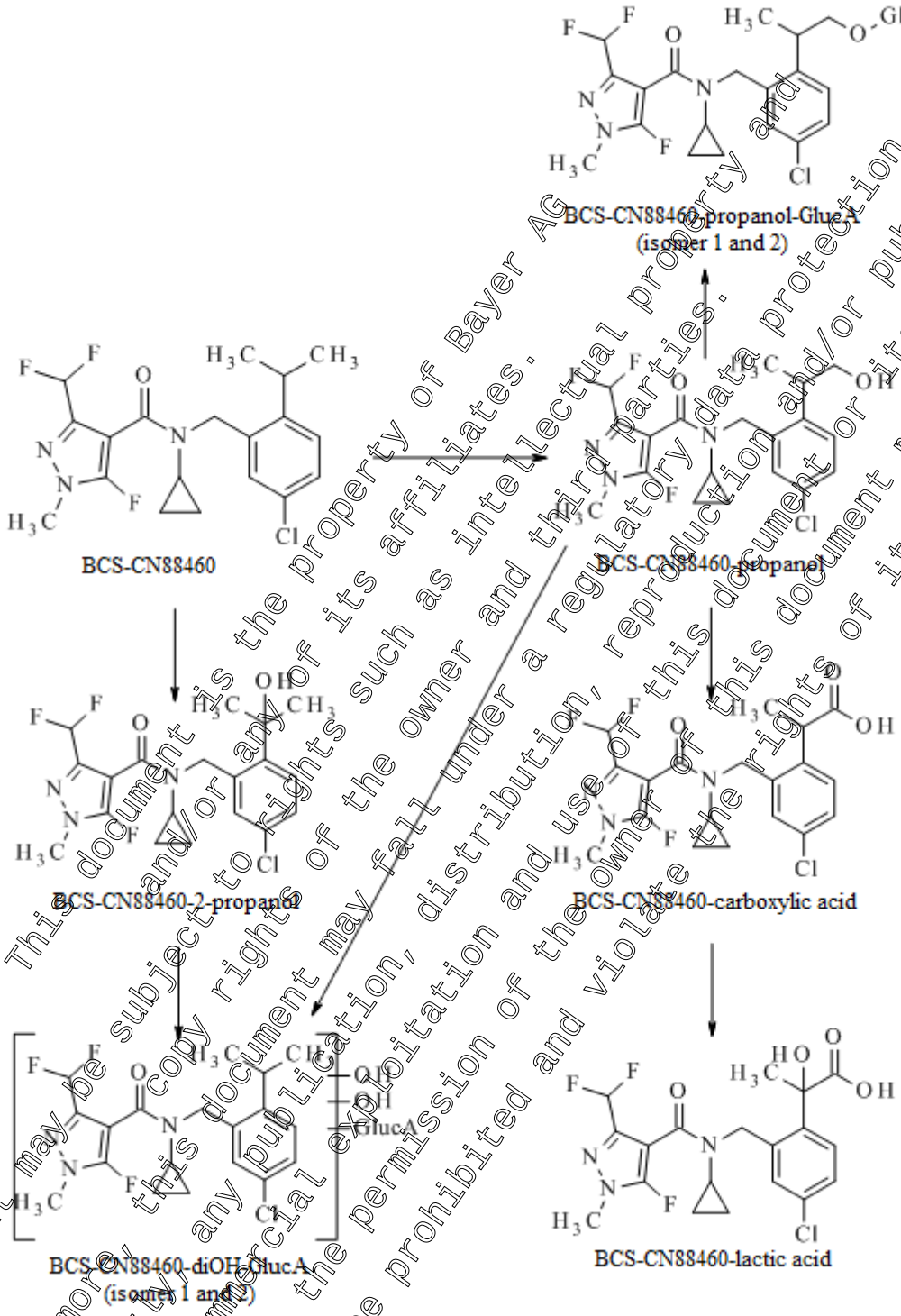
Figure 5.1.1- 2: Proposed metabolic pathway of [phenyl-UL-^{14}C]BCS-CN88460 in the rat (Part A)



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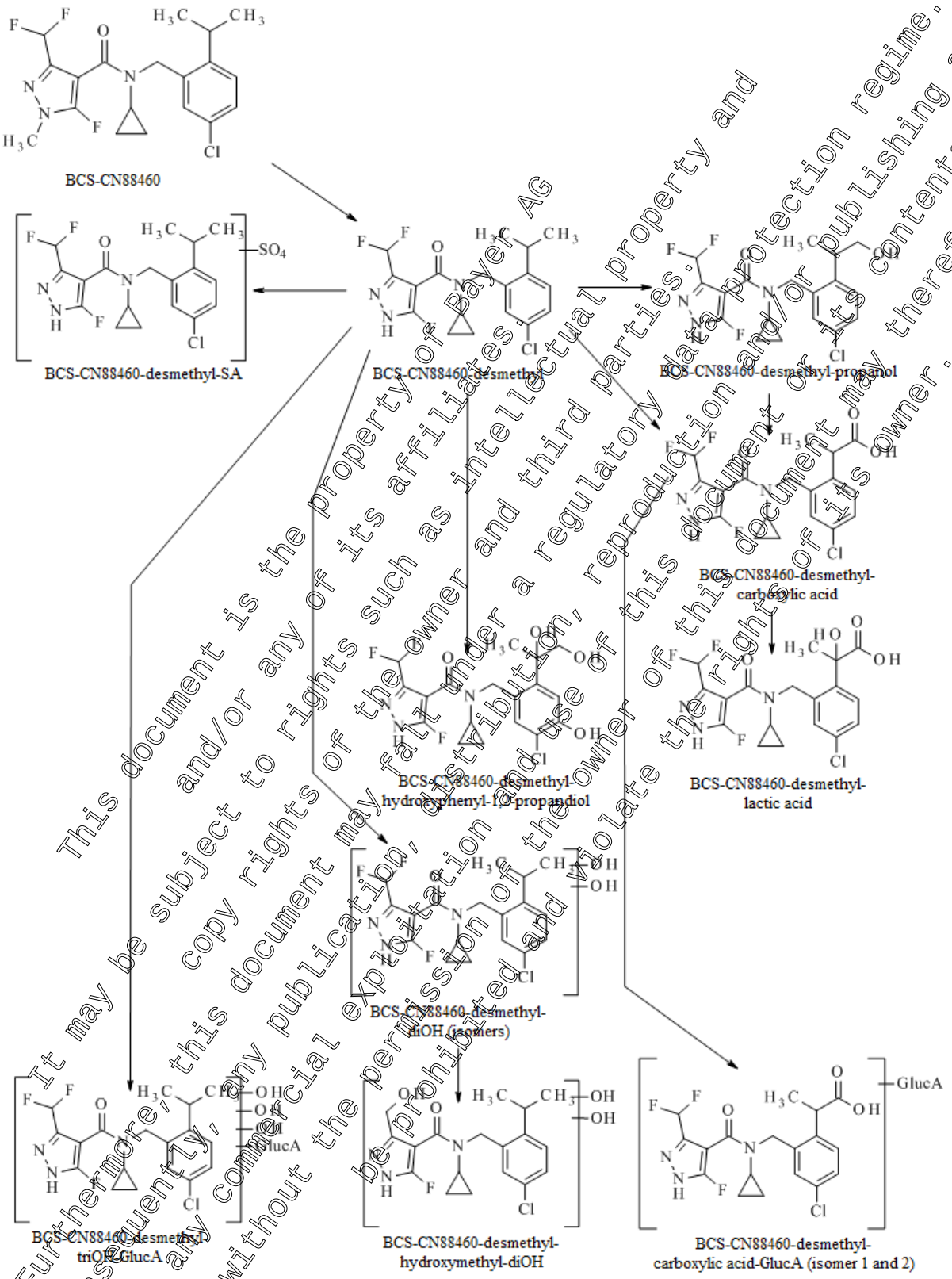
Part B of the pathway is presented on the next page

Figure 5.1.1- 2: Proposed metabolic pathway of [phenyl-UL-^{14}C]BCS-CN88460 in the rat (Part B)



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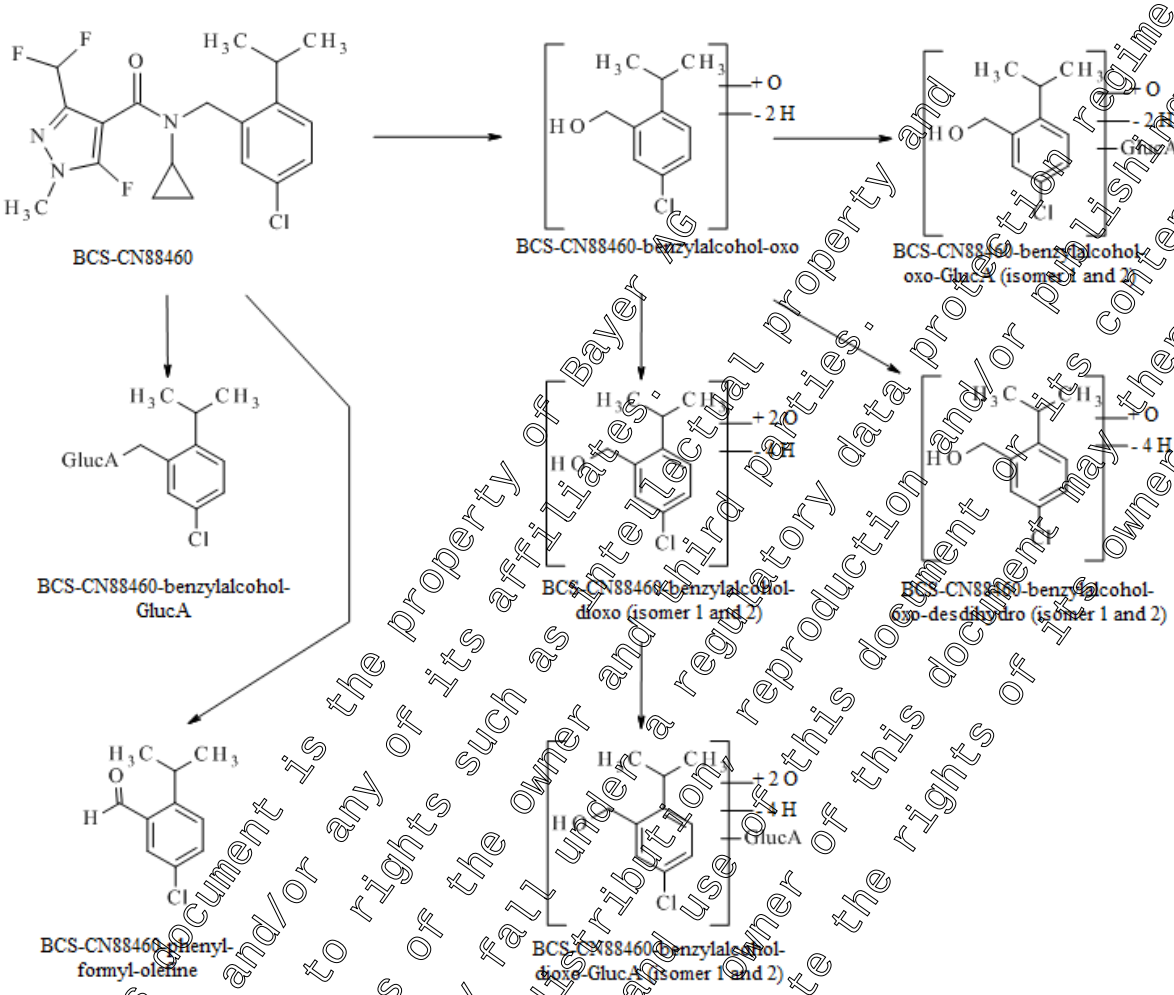
Part C of the pathway is presented on the next page

Figure 5.1.1- 2: Proposed metabolic pathway of [phenyl-UL-^{14}C]BCS-CN88460 in the rat (Part C)



Report: KCA 5.1.1/03; [redacted] R.; [redacted], N.; 2017; M-602456-01-1
Title: [Pyrazole-4-14C]BCS-CN88460: Distribution of the total radioactivity in male and female rats determined by quantitative whole body autoradiography, determination of the exhaled 14CO2, and pilot metabolism experiments
Report No.: EnSa-16-1012
Document No.: M-602456-01-1
Guideline(s): Regulation (EC) No 1107/2009 amended by Commission Regulation (EU) No 283/2013
OECD Guideline for the Testing of Chemicals, 417
US EPA OCSPP Health Effects Test Guidelines, 870.7485
Japan/FAMIC-ACIS Notification 12 Nousan 8147
Guideline deviation(s): not specified
GLP/GEP: **yes**

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Executive Summary

The toxicokinetic behaviour (absorption, distribution, and excretion) and metabolism of BCS-CN88460, labelled with ^{14}C in pyrazole-4 moiety of the molecule, was investigated in male and female Wistar rats. The rats received a single mean target dose of about 5 mg of the test compound per kilogram body weight by oral gavage.

The absorption of radioactivity from the GI-tract, distribution to and elimination from blood, organs and tissues were analysed qualitatively and quantitatively by whole body autoradioluminography (WBAL). For both sexes, one rat each was taken for cryosectioning at 1, 4, 8, 24, 48, 72, 120, and 168 h after administration (Test 2: male; Test 3: female). The amounts of radioactivity in excreta (urine and faeces) and exhaled carbon dioxide were additionally determined for selected time periods.

To investigate the metabolism, one male rat each was sacrificed at 1, 4, 8, and 24 h after administration (Test 1). Samples of urine, faeces, plasma, liver, kidney, and thyroid were collected and afterwards prepared for the chromatographic evaluation of the metabolic profiles by HPLC with radiometric detection.

[Pyrazole-4-^{14}C]BCS-CN88460 was obviously absorbed very fast from the gastrointestinal tract of male and female Wistar rats after single oral administration leading to maximum plasma levels in the systemic compartment blood 1 h after dosing. More than 80% of the radioactivity was excreted *via* the faeces and about 14% with urine. The excretion was nearly completed within 3 days.

Less than 0.01% of the given dose was exhaled during a sampling period for the animals of both sexes. This demonstrates that the ^{14}C-labelling in the pyrazole-4 moiety of the molecule was stable with regard to formation of carbon dioxide.

The absorbed test compound related radioactivity was distributed throughout the animal bodies immediately after dosing with a clear preference to the liver and kidney that are the responsible organs for metabolism (liver) and excretion (kidney). In case of females additionally a preference to the myocardium, some glandular organs (e.g. harderian gland), and fatty tissues was observed.

Maximum concentrations of radioactivity were reached 1 h after dosing in all organs and tissues in both sexes, except for vitreal body and renal medulla from female rats. The radioactivity was almost completely and rapidly eliminated from the central and peripheral compartments of the animals until sacrifice. At that time trace amounts of radioactivity were detected only in blood, liver, kidney, lung, adrenal gland, and nasal mucosa of male rats, as well as in blood, liver, and nasal mucosa of female rats. This shows that the remaining residual radioactivity is excreted predominantly by the faecal route smoothly from the animal bodies.

No relevant sex related differences concerning the maximum equivalent concentrations in blood, organs, and tissues was observed in male or female rats. The excretion *via* urine and faeces was slightly faster in male rats.

The results of the WBA indicate that male and female rats exhibit a very similar absorption, distribution, and excretion behaviour. Any accumulation or substantial retention of [pyrazole-4-^{14}C]BCS-CN88460 related residues in organs and tissues of male and female rats can be excluded.

The results of the pilot metabolism experiment with male rats (Test 1) confirm basically the observations made by the WBA referring to absorption and excretion. Total radioactivity of given dose was excreted up to 12.89% *via* urine and up to 81.02% *via* faeces of male rats within the 24 h experimental period.

Urine samples were analysed without any sample preparation. Faeces were conventionally extracted (3 times ACN/water (8+2, v/v)) and the extraction rates amounted to 62.7% for samples collected during a period of 0-8 h, and to 89.6% for samples collected during a period of 0-24 h following dosing. Plasma samples were analysed after protein precipitation. The total radioactivity in plasma samples was calculated based on LSC measurements and decreased from 2.579 mg/kg at 1 h to 0.093 mg/kg at 24 h after dosing. Total radioactivity of liver, kidney, and thyroid samples was

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determined by LSC as sum of the conventional extracts (3 times ACN/water (8/2; v/v)) and the post extraction solids. The TRR in liver samples decreased from 11.787 mg eq/kg (animal 264) at 1 h after administration to 0.407 mg eq/kg (animal 267) at 24 h after administration. Total radioactivity in kidney samples decreased from 3.222 mg eq/kg (animal 264) at 1 h to 0.110 mg eq/kg h (animal 267) at 24 h post administration. For the combined thyroid samples from male rats over all sampling points a value of 0.588 mg eq/kg was determined.

Parent compound and metabolites were quantified by HPLC in the metabolite profiles from urine, samples of plasma, and from extracts of liver, kidney, and faeces. Due to the low of radioactivity metabolites could not be quantified in the profile of the extract of thyroid.

Parent compound and up to twelve metabolites were identified in isolated fractions from urine and extract of faeces (both 0-24 h) by LC-MS/MS and NMR. The peaks were assigned in the HPLC profiles of urine and faeces extract, respectively. In addition peaks of chromatograms in plasma samples and extracts of liver and kidney were assigned by comparison of their retention time or by HPLC co-chromatography with the isolated fraction based on the profiling method. All other ^{14}C-signals in the respective HPLC chromatograms were assigned as 'unknown'. They were characterised by their retention times.

In urine samples parent compound was not identified. Approximately 13% of the unchanged parent compound had been excreted via faeces.

Beside parent compound the following metabolites have been identified:

- BCS-CN88460-pyrazole-carboxylic acid
- BCS-CN88460-cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1)
- BCS-CN88460-cyclopropyl-pyrazole-carboxamide
- BCS-CN88460-desmethyl-hydroxymethyl-diOH
- BCS-CN88460-desmethyl-hydroxyphenyl-1,2-propandiol
- BCS-CN88460-desmethyl-hydroxyphenyl-2-propanol
- BCS-CN88460-desmethyl-lactic acid
- BCS-CN88460-desmethyl-hydroxymethyl-carboxylic acid
- BCS-CN88460-lactic acid
- BCS-CN88460-desmethyl-carboxylic acid
- BCS-CN88460-carboxylic acid
- BCS-CN88460-desmethyl

Parent compound was not detected in plasma samples. The most prominent components in plasma were BCS-CN88460-desmethyl-carboxylic acid (0.885 mg/kg at 1 h), BCS-CN88460-cyclopropyl-pyrazole-carboxamide (0.546 mg/kg at 4 h) and BCS-CN88460-carboxylic acid (0.277 mg/kg; 1 h). The metabolite pattern in the stored plasma samples was demonstrated to be stable up to 1 year after sampling.

In liver samples up to 35 metabolites were detected beside the parent compound. The most prominent components identified and quantified at 1 h after administration were BCS-CN88460-carboxylic acid (2.580 mg/kg), BCS-CN88460-desmethyl-carboxylic acid (1.749 mg/kg), and BCS-CN88460-lactic acid (0.895 mg/kg). BCS-CN88460-desmethyl lactic acid showed the highest concentration at 4 h after administration (1.144 mg/kg).

Beside the parent compound up to 20 metabolites were detected in kidney samples. The most prominent components identified and quantified were BCS-CN88460-carboxylic acid (0.567 mg/kg), BCS-CN88460-desmethyl-carboxylic acid (0.539 mg/kg), and BCS-CN88460-cyclopropyl-pyrazole-carboxamide (0.237 mg/kg) with highest values at 1 h after administration.

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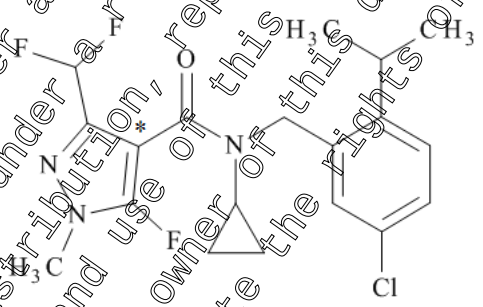
The principal metabolic reactions of [pyrazole-4-^{14}C]BCS-CN88460 in the rat are listed below:

- demethylation of the pyrazole moiety
- hydroxylation in position 1 and position 2 of the propyl group, in the phenyl ring and after defluorination of the difluoromethyl moiety was leading to mono-, di-, or tri-hydroxy compounds. Hydroxylation in other positions was also detected, but was not exactly located by structure elucidation.
- further oxidation of the 1-propanol group was leading to a carboxylic acid group or in combination with a 2-propanol group to a lactic acid group
- cleavage of the phenyl moiety was leading to BCS-CN88460-cyclopropyl-pyrazole-carboxamide
- cleavage of the phenyl moiety in combination with cleavage of the cyclopropyl ring was leading to BCS-CN88460-pyrazole-carboxylic acid
- conjugation of BCS-CN88460-cyclopropyl-pyrazole-carboxamide with glucuronic acid

I. Material and Methods

A. Material

1. Test material:

Test substance	
Chemical structure	
	*denotes the position of the ^{14}C-radiolabel
Radiolabelled test material	[Pyrazole-4-^{14}C]BCS-CN88460
Specific radioactivity	3.90 MBq/mg = 2.34 x 10^{8} dpm/mg
Chemical purity	>99% (HPLC)
Radiochemical purity	>99% (HPLC); >99% (TLC)
Vehicle	0.5%-aqueous Tragacanth solution
Preparation of dosing solution	radiolabelled test compound suspended in aqueous Tragacanth solution

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2. Test Animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	Wistar Hsd/Cpb: WU
Breeding facility:	[redacted]
Sex and numbers involved:	Males: 4 animals for Test 1 (pilot metabolism) Males: 8 animals for Test 2 (QWBA) Females: 8 animals for Test 3 (QWBA)
Age:	about 6-7 weeks (male rats) and about 8-9 weeks (female rats) at the time of delivery
Body weight:	Males of Test 1: 230 - 253 g at the time of administration 228 - 243 g at the time of sacrifice Males of Test 2: 196 - 211 g at the time of administration 192 - 204 g at the time of sacrifice Females of Test 3: 194 - 204 g at the time of administration 197 - 211 g at the time of sacrifice
Acclimatization:	Makrolon® cages on wood shavings in the test facility for about 7 days prior to the administration.
Identification:	Cage cards on which the study number, test compound name and individual animal number were displayed. Additional labelling with water-insoluble spots on the tail
Housing:	After administration of the radiolabelled test substance individually in Makrolon® metabolism cages under conventional hygienic conditions in air-conditioned rooms Temperature 21 - 24 °C, relative humidity 20 - 63 %. 12 / 12 hours light / dark cycle.
Feed and water:	Rat/mice maintenance long life diet (article no. V1534-000), supplied by [redacted] (ca. 16 g per animal and day) Last feeding ca. 16 h prior to dosing Next feeding ca. 6 h after dosing Tap water from municipal water supply, *ad libitum*

B. Study design

1. Dosing

Animals were assigned to their respective test groups 1, 2 and 3. Four male rats were dosed in Test 1, 8 male rats were dosed in Test 2, and 8 female rats were dosed in Test 3. The suspension was administered to the rats by oral gavage using a syringe attached to an animal-feeding knob cannula. Each rat received 2 mL of the respective administration suspension of [pyrazole-4-^{14}C]BCS-CN88460. The volume was based on a nominal average animal weight of 200 g. The concentration of each administration suspension was calculated to reach a target dose of 5 mg per kg body weight of the rats. Due to different animal weights at administration, the mean actual doses in the tests ranged from 4.27 mg/kg to 5.15 mg/kg body weight. The stability of the test compound in the suspensions was assured by radio-HPLC analysis after administration.

2. Sacrifice

The rats of Test 1 (pilot metabolism) were sacrificed in deep pentobarbital sodium anaesthesia by transection of the cervical vessels and exsanguination. The rats of Test 2 and Test 3 (QWBA) were sacrificed by an overdose of pentobarbital sodium anaesthesia.

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3. Quantitative whole-body autoradiography

The distribution of total radioactivity without differentiation of unchanged test compound and radiolabelled biotransformation products in, and the elimination from blood, organs, and tissues was determined qualitatively and quantitatively at various time-points by WBAL.

For WBAL the sacrificed animals were deep frozen at about -70 °C and embedded in CMC (carboxymethylcellulose). Sagittal sections (from cranial to caudal) were cut with a thickness of 50 µm at about -25 °C using a cryomicrotome and the sampled slices were dehydrated for at least 24 h. The distribution of the radioactivity in the whole body sections was detected employing the Fuji BAS 5000® phosphor-imaging system. The quantification of equivalent concentrations in the organ and tissues was performed using ^{14}C-spiked blood standards for calibration.

4. Collection of excreta

After administration of the radiolabelled test compound, the rats were kept individually in Makrolon® metabolism cages, which allowed for separate and quantitative collection of urine, faeces, and expired air. Urine was collected separately for each animal in a cryogenic trap cooled with dry ice in intervals of 1h, 4 h, 8 h, 24 h (Test 1) or in intervals of 1h, 4 h, 8 h, 24 h and every 24 h until 168 h (Test 2 and Test 3). The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction. The radioactivity was determined by LSC.

The faeces samples of Test 1 were collected from two individual animals in intervals of 0-8 h and 0-24 h. The radioactivity was calculated after extraction from the sum of extracts (determined by LSC) and post extraction solids (determined by combustion/LSC). The faeces samples of Test 2 and Test 3 were collected every 24 h separately for each animal. Each faeces sample was mixed approximately 1:1 with water, then weighed and homogenised. The radioactivity was determined by combustion/LSC.

5. Trapping of expired air (Test 2 and Test 3)

Carbon dioxide and other volatiles in the expired air were collected from four male and four female animals for the time ranges 0 - 24 h and 24 - 48 h. The respective metabolism cages were attached to a high velocity air pump and ventilated with ca. 3 L of air per minute and cage. The air was passed through a trapping system of two gas-washing bottles each containing about 250 - 300 mL of a 1:1-mixture of ethanolamine/ethanol. At sampling, the exact volume was determined, from which an aliquot was taken for the determination of radioactivity by LSC.

6. Preparation of blood/plasma samples (Test 2)

Blood micro-samples up to 168 h were collected at different time points by pressing a capillary coated with heparin in a small cut in the tail vein. The wound was closed with adhesive tape. The capillaries were centrifuged for about 10 min using a haematocrit centrifuge to separate plasma from the blood cells (mainly erythrocytes). After centrifugation, the capillary was broken at the border between plasma and blood cells. The plasma was weighed and used for radioactivity measurement by LSC.

7. Plasma and organs at sacrifice (Test 1)

Following sacrifice, the oozing out individual blood samples were collected from the cut wounds in heparinized test tubes. Blood was separated into plasma and blood cells (mainly erythrocytes) by centrifugation. After weighing subsamples from plasma were taken for radioactivity measurement by LSC. The residual samples were kept frozen until analytical investigations.

The individual organs (liver, kidney, and thyroid) were weighed immediately after dissection. To keep enough sample material for extraction and for the metabolic profiling, no determination of radioactivity was performed in these organs. All samples were kept frozen until extraction and analytical investigations. The total radioactivity was calculated after extraction from the sum of extracts (determined by LSC) and post extraction solids (determined by combustion/LSC).

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

C. Analytical procedures
Samples were analysed by radiochromatographic (HPLC) and spectroscopic (LC-MS/MS, ^{1}H-NMR) methods.

1. Preparation of samples, extraction and analysis (Test 1)
The collected plasma of each sampling point (1, 4, and 8 h) was analysed by HPLC. Plasma proteins were precipitated prior to HPLC analysis. Urine samples were used for metabolic profiling without further sample preparation. The faeces samples of the sampling intervals 0-8 h and 0-24 h and liver, kidney and pooled thyroid samples were extracted with acetonitrile/water (8/2; v/v) mixtures using a homogeniser and used for the quantitative analysis of parent compound and metabolites by HPLC.

The storage stability of plasma was monitored for a storage period (at ≤ -18 °C) of up to 1 year after sacrifice. Metabolite profiles were recorded directly after sampling and in addition they were reanalysed after 3 months, 6 months and 1 year after sampling. The metabolite pattern in the stored plasma samples was demonstrated to be stable during the time of investigation.

2. Identification / characterisation and quantification of residues (Test 1)
Parent compound and its metabolites in samples from plasma, urine and in extracts from liver, kidney and faeces were quantified by HPLC based on reversed phase chromatography using an acidic water/acetonitrile gradient. Metabolites in the profile of the extract of thyroid could not be quantified, due to the low amount of radioactivity.

Parent compound and metabolites were identified in isolated fractions from urine and extract of faeces (both 0-24 h) by LC-MS/MS and NMR. They were assigned in the profiles of urines and extracts of plasma, liver, kidney and faeces by comparison of the retention times or by HPLC co-chromatography with the isolated fractions. In addition the identified metabolites were used as reference compounds in the rat ADME studies.

II. RESULTS AND DISCUSSION

The toxicokinetic behaviour of [pyrazole-4-^{14}C]BCS-CN88460 was investigated in male and female Wistar rats after a single oral administration at a dose level of 5 mg/kg body weight.
The absorption of radioactivity from the GI-tract, distribution to, and elimination from blood, organs, and tissues were analysed qualitatively and quantitatively by WBAL (Test 2 and Test 3). One rat each was taken for cryosectioning at 1, 4, 8, 24, 48, 72, 120, and 168 h after administration. The amounts of radioactivity in the excreta (urine and faeces) and exhaled carbon dioxide were determined for selected time periods
For investigation of the metabolism (Test 1) one male rat each was sacrificed at 1, 4, 8, and 24 h after administration. Samples of urine, faeces, plasma, liver, kidney and thyroid were analysed by HPLC.

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A. Toxicokinetic behaviour of male rats (Test 2)

Distribution of radioactivity to and elimination from organs and tissues

For all organs and tissues of male rats (Test 2), maximum concentrations were reached at one hour after administration. At this time, the organ/blood concentration ratio values were highest for liver (factor 6.8) and kidney (factor 1.6). The ratios for the glandular organs ranged from 0.75 (pituitary gland) to 1.0 (adrenal gland) and for brown fat and the myocardium a value of about 1.0 was calculated. For all other organs and tissues, the values ranged from 0.17 (vitreal body) to 0.76 (lung). The test compound related radioactivity was cleared from blood very fast and distributed to the entire body. Compared to C_{max}, the equivalent concentrations declined in nearly all organs and tissues by a factor of approximately 11.8 (nasal mucosa) to 73.3 (myocardium) within 24 h after dosing. For brain, spinal cord and harderian gland, the values were already < LOQ at this time.

Residual concentrations in organs and tissues

Until the end of the test, 168 h after dosing, equivalent concentrations declined to values < LOQ for most organs and tissues. Trace amounts of radioactivity were detected in blood, liver, kidney, lung, adrenal gland and nasal mucosa, only. The equivalent concentrations ranged from 0.46% (liver) to 3.56% (nasal mucosa) compared to C_{max}.

Time course of radioactivity concentration in plasma

The maximum plasma level (C_{max}) was reached 1 h after administration. At this time point, the radioactivity levels corresponded to about 47% of the equidistribution concentration (C_{norm} = 0.468). The plasma concentrations declined to about 26% within 7 h and to about 2% of C_{max} within 48 h. At 168 h a very low value of about 0.4% of C_{max} was calculated.

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Table 5.1.1- 29: Distribution of radioactivity in blood, organs and tissues of male rats (Test 2) after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw

	Equivalent concentration CEQ [µg eq/g]							
	Animal no.							
	268	269	270	271	272	273	274	275
	Time of sacrifice [hours after administration]							
Organ or tissue	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h
Blood	***1.244***	0.638	0.246	0.054	0.032	0.059	0.055	0.027
Liver	***8.437***	2.814	1.415	0.286	0.213	0.153	0.098	0.039
Renal cortex	***1.730***	0.763	0.288	0.050	0.030	0.030	0.021	0.014
Renal medulla	***2.160***	1.034	0.381	0.056	0.028	0.022	0.021	0.010
Kidney total	***1.945***	0.898	0.332	0.053	0.029	0.026	0.021	0.012
Brown fat	***1.230***	0.513	0.178	0.026	0.013	0.016	0.014	---
Perirenal fat	***0.503***	0.263	0.097	0.032	---	0.009	< LOQ	---
Skeleton muscle	***0.427***	0.180	0.063	0.007	< LOQ	< LOQ	< LOQ	< LOQ
Myocardium	***1.168***	0.453	0.173	0.016	0.008	0.009	0.008	< LOQ
Lung	***0.943***	0.491	0.159	0.023	0.013	0.008	0.013	0.007
Spleen	***0.496***	0.206	0.078	0.011	0.007	0.008	0.008	< LOQ
Pancreas	***0.805***	0.303	0.096	0.014	0.006	0.006	< LOQ	---
Bone marrow	***0.455***	0.210	0.096	0.009	---	---	---	---
Testis	***0.419***	0.313	0.114	0.012	< LOQ	< LOQ	< LOQ	< LOQ
Brain	***0.382***	0.170	0.050	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Spinal cord	***0.403***	0.173	0.054	< LOQ	---	---	---	---
Pituitary gland	***0.933***	0.316	0.125	0.019	---	---	---	---
Pineal body	***0.836***	0.263	0.144	0.015	---	---	---	---
Adrenal gland	***1.243***	0.475	0.190	0.029	0.012	0.017	0.021	0.009
Thymus	***0.457***	0.191	0.077	0.008	< LOQ	< LOQ	< LOQ	< LOQ
Thyroid gland	***0.989***	---	0.070	0.019	---	0.013	---	---
Salivary gland	***0.949***	0.325	0.120	0.017	0.008	0.011	0.007	---
Nasal mucosa	***0.555***	---	0.145	0.047	0.047	0.045	0.028	0.020
Vitreal body	***0.207***	0.133	0.056	0.013	< LOQ	< LOQ	< LOQ	< LOQ
Harderian gland	***0.947***	---	---	---	---	---	---	---

--- : Organ or tissue was visible in the rat sections but not discernible in the radioluminograms.
CEQ : Equivalent concentration [µg eq/g]
Maximum CEQ - values (= CEQmax) are shown in italics and bold style.

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Table 5.1.1- 30: Toxicokinetic parameters derived from individual concentration *vs.* time data of radioactivity in blood, organs, and tissues of male rats (Test 2) after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw

Organ or tissue	CEQ_{max}	CEQ_{max} ratio organ/blood	t_{max} [h]	$CEQ_{168\ h}$	$CEQ_{168\ h}$ ratio organ/blood
Blood	1.244	1.00	1	0.027	1.00
Liver	8.437	**6.78**	1	0.039	**1.41**
Renal cortex	1.730	**1.39**	1	0.014	0.51
Renal medulla	2.160	**1.74**	1	0.010	0.38
Kidney total	1.945	**1.56**	1	0.012	0.44
Brown fat	1.230	0.99	1	---	n.c.
Perirenal fat	0.503	0.40	1	---	n.c.
Skeleton muscle	0.427	0.34	1	< LOQ	n.c.
Myocardium	1.168	0.94	1	< LOQ	n.c.
Lung	0.943	0.76	1	0.007	0.24
Spleen	0.496	0.40	1	< LOQ	n.c.
Pancreas	0.805	0.65	1	---	n.c.
Bone marrow	0.458	0.37	1	< LOQ	n.c.
Testis	0.419	0.34	1	< LOQ	n.c.
Brain	0.382	0.31	1	< LOQ	n.c.
Spinal cord	0.403	0.32	1	---	n.c.
Pituitary gland	0.933	0.75	1	---	n.c.
Pineal body	0.836	0.67	1	---	n.c.
Adrenal gland	1.243	1.00	1	0.009	0.34
Thymus	0.457	0.37	1	< LOQ	n.c.
Thyroid gland	0.989	0.79	1	---	n.c.
Salivary gland	0.949	0.76	1	---	n.c.
Nasal mucosa	0.555	0.45	1	0.020	0.72
Vitreal body	0.207	0.17	1	< LOQ	n.c.
Harderian gland	0.947	0.76	1	---	n.c.

$$\text{Ratio} = \frac{CEQ_{organ}}{CEQ_{blood}}$$

CEQ: Equivalent concentration [µg eq/g]
t_{max}: Time at which the maximum radioactivity concentration occurred in blood, organs and tissues following administration of an extravascular dose.
n.c.: not calculated because either TRR-values were < LOQ or organ/tissue was not discernible in the radioluminograms

bold style = values ≥1.0

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Table 5.1.1- 31: Percentage ratio of the individual equivalent concentrations in the blood, organs, and tissues over the maximum concentrations (CEQ_{max}) of male rats (Test 2) after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw

	Ratio [%] of the individual CEQ-values in the blood, organs and tissues over CEQ_{max}							
	Animal no.							
	268	269	270	271	272	273	274	275
	Time of sacrifice [hours after administration]							
Organ or tissue	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h
Blood	100.00	51.31	19.77	4.35	2.57	4.73	4.42	2.21
Liver	100.00	33.36	16.78	3.39	2.53	1.81	1.17	0.46
Renal cortex	100.00	44.09	16.34	2.89	1.73	1.73	1.23	0.81
Renal medulla	100.00	47.86	17.66	2.61	1.29	1.02	0.97	0.48
Kidney total	100.00	46.19	17.07	2.73	1.49	1.33	1.09	0.63
Brown fat	100.00	41.66	14.24	2.15	1.07	1.31	1.16	n.c.
Perirenal fat	100.00	52.31	19.32	6.39	n.c.	1.73	n.c.	n.c.
Skeleton muscle	100.00	42.29	14.75	1.62	n.c.	n.c.	n.c.	n.c.
Myocardium	100.00	38.74	14.76	1.36	0.67	0.81	0.66	n.c.
Lung	100.00	52.05	16.86	2.42	1.34	0.88	1.38	0.70
Spleen	100.00	41.43	15.66	2.14	1.41	1.59	1.54	n.c.
Pancreas	100.00	37.59	11.92	1.73	0.78	0.71	n.c.	n.c.
Bone marrow	100.00	46.10	20.99	1.90	n.c.	n.c.	n.c.	n.c.
Testis	100.00	74.82	27.33	2.79	n.c.	n.c.	n.c.	n.c.
Brain	100.00	44.48	13.14	n.c.	n.c.	n.c.	n.c.	n.c.
Spinal cord	100.00	42.96	13.34	n.c.	n.c.	n.c.	n.c.	n.c.
Pituitary gland	100.00	33.82	13.39	2.00	n.c.	n.c.	n.c.	n.c.
Pineal body	100.00	31.42	13.66	1.74	n.c.	n.c.	n.c.	n.c.
Adrenal gland	100.00	38.25	15.27	2.33	0.97	1.40	1.68	0.76
Thymus	100.00	41.78	16.75	1.83	n.c.	n.c.	n.c.	n.c.
Thyroid gland	100.00	n.c.	7.11	1.97	n.c.	1.34	n.c.	n.c.
Salivary gland	100.00	34.25	12.65	1.82	0.85	1.12	0.73	n.c.
Nasal mucosa	100.00	n.c.	26.05	8.47	8.46	8.05	5.00	3.56
Vitreal body	100.00	64.23	27.04	6.38	n.c.	n.c.	n.c.	n.c.
Harderian gland	100.00	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.

$$\text{Ratio} = \frac{CEQ_{indiv.\ time}}{CEQ_{max}} \times 100\ [\%]$$

CEQ: Equivalent concentration [µg eq/g]
n.c.: not calculated because either TRR-values were < LOQ or organ/tissue was not discernible in the radioluminograms

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Table 5.1.1- 32: Equivalent and dose normalised concentrations of plasma microsamples of male rats (Test 2) after a single oral administration of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw

Sampling time [h p admin.]	Equiv. concentration C [µg eq/g]	Dose normalised concentration C_{norm} *)
0.25	0.704	0.136
0.5	2.207	0.427
1	2.418	0.468
2	1.961	0.379
7	0.637	0.123
24	0.095	0.018
48	0.049	0.009
72	0.034	0.007
96	0.025	0.005
120	0.020	0.004
144	0.016	0.003
168	0.012	0.002

*) administered dose: 5.17 mg/kg bw

B. Toxicokinetic behaviour of female rats (Test 3)

Distribution of radioactivity to and elimination from organs and tissues

In case of female rats (Test 3), the maximum concentrations for almost all organs and tissues were reached between one to four hours after administration. At this time, the organ/blood concentration ratio values were highest for liver (factor 8.1), brown fat (factor 2.7) and kidney (factor 1.89). The ratios for the glandular organs ranged from 1.1 (pituitary gland) to 2.1 (harderian gland) and for perirenal fat, myocardium and ovary values of about 1.4, 1.9, and 1.1, respectively. For all other organs and tissues, the values ranged from 0.21 (vitreal body) to 0.82 (lung).
The radioactivity was cleared from blood very fast and distributed to the entire body. Compared to Cmax, the equivalent concentrations declined in nearly all organs and tissues by factors between 12.8 (nasal mucosa) to 71.6 (spinal cord) within 24 h after dosing. For bone marrow and pineal body, the values were below LOQ at 24 h after dosing.

Residual concentrations in organs and tissues

Until the end of the test, 168 h after dosing, equivalent concentrations declined to values below LOQ for most organs and tissues of female rats. Only in blood, liver and nasal mucosa trace amounts of radioactivity were detected. For these organs and tissues, the equivalent concentrations were 0.22% (liver), 1.69% (blood, and 2.26% (nasal mucosa).

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Table 5.1.1- 33: Distribution of radioactivity in blood, organs and tissues of female rats (Test 3) after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw

	Equivalent concentration CEQ [µg eq/g]							
	Animal no.							
	276	277	278	279	280	281	282	283
	Time of sacrifice [hours after administration]							
Organ or tissue	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h
Blood	***0.754***	0.719	0.280	0.052	0.031	0.011	0.010	0.013
Liver	***6.132***	5.043	2.482	0.411	0.127	0.026	0.016	0.014
Renal cortex	***1.224***	0.964	0.376	0.050	0.027	---	---	---
Renal medulla	1.594	***1.824***	0.565	0.090	0.047	---	---	---
Kidney total	***1.409***	1.394	0.467	0.070	0.037	---	---	---
Brown fat	***2.048***	0.966	0.583	0.048	0.018	---	---	---
Perirenal fat	***1.025***	0.669	0.460	0.069	0.023	0.012	---	---
Skeleton muscle	***0.717***	0.206	0.110	0.010	< LOQ	< LOQ	---	---
Myocardium	***1.423***	0.749	0.403	0.041	0.011	LOQ	< LOQ	---
Lung	***0.622***	0.587	0.251	0.022	0.021	< LOQ	< LOQ	< LOQ
Spleen	***0.483***	0.284	0.124	0.014	0.006	< LOQ	< LOQ	---
Pancreas	***0.942***	0.520	0.239	---	0.008	LOQ	---	---
Bone marrow	***0.440***	0.324	0.104	---	---	---	---	---
Ovary	***0.854***	0.593	0.242	0.030	0.013	---	---	---
Uterus	***0.593***	0.550	0.223	0.042	0.035	< LOQ	---	---
Brain	***0.438***	0.138	0.088	0.007	< LOQ	< LOQ	< LOQ	---
Spinal cord	***0.538***	0.161	0.095	0.008	< LOQ	---	---	---
Pituitary gland	***0.827***	0.429	0.240	0.028	---	---	---	---
Pineal body	***0.817***	0.470	0.215	---	---	---	---	---
Adrenal gland	***1.344***	0.844	0.395	0.045	0.020	0.009	---	---
Thymus	***0.406***	0.249	0.119	0.011	< LOQ	< LOQ	< LOQ	---
Thyroid gland	***0.900***	0.597	0.258	0.031	0.013	---	---	---
Salivary gland	***0.950***	0.349	0.230	0.025	0.008	< LOQ	---	---
Nasal mucosa	***0.473***	0.351	0.181	0.037	0.019	0.010	0.010	0.011
Vitreal body	0.056	***0.161***	0.034	0.013	< LOQ	< LOQ	---	---
Harderian gland	***1.593***	0.639	0.373	0.038	0.014	< LOQ	---	---

--- : Organ or tissue was visible in the rat sections but not discernible in the radioluminograms.
CEQ : Equivalent concentration [µg eq/g]
Maximum CEQ - values (= CEQmax) are shown in italics and bold style.

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Table 5.1.1- 34: Toxicokinetic parameters derived from individual concentration *vs.* time data of radioactivity in blood, organs, and tissues of female rats (Test 3) after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw

Organ or tissue	CEQ_{max}	CEQ_{max} ratio organ/blood	t_{max} [h]	$CEQ_{168\ h}$	$CEQ_{168\ h}$ ratio organ/blood
Blood	0.754	1.00	1	0.013	1.00
Liver	6.132	**8.13**	1	0.014	**1.06**
Renal cortex	1.224	**1.62**	1	---	n.c.
Renal medulla	1.824	**2.42**	4	---	n.c.
Kidney total	1.409	**1.87**	1	---	n.c.
Brown fat	2.048	**2.72**	1	---	n.c.
Perirenal fat	1.025	**1.36**	1	---	n.c.
Skeleton muscle	0.717	0.95	1	---	n.c.
Myocardium	1.423	**1.89**	1	---	n.c.
Lung	0.622	0.82	1	< LOQ	n.c.
Spleen	0.483	0.64	1	---	n.c.
Pancreas	0.942	**1.25**	1	---	n.c.
Bone marrow	0.440	0.58	1	---	n.c.
Ovary	0.854	**1.13**	1	---	n.c.
Uterus	0.593	0.79	1	---	n.c.
Brain	0.438	0.58	1	---	n.c.
Spinal cord	0.538	0.71	1	---	n.c.
Pituitary gland	0.827	**1.10**	1	---	n.c.
Pineal body	0.817	**1.08**	1	---	n.c.
Adrenal gland	1.344	**1.78**	1	---	n.c.
Thymus	0.406	0.54	1	---	n.c.
Thyroid gland	0.900	**1.19**	1	---	n.c.
Salivary gland	0.950	**1.26**	1	---	n.c.
Nasal mucosa	0.473	0.63	1	0.011	0.84
Vitreal body	0.161	0.21	4	---	n.c.
Harderian gland	1.593	**2.11**	1	---	n.c.

$$\text{Ratio} = \frac{CEQ_{organ}}{CEQ_{blood}}$$

CEQ: Equivalent concentration [µg eq/g]
t_{max}: Time at which the maximum radioactivity concentration occurred in blood, organs and tissues following administration of an extravascular dose.
n.c.: not calculated because either TRR-values were <LOQ or organ/tissue was not discernible in the radioluminograms
bold style = values >1.0

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Table 5.1.1- 35: Percentage ratio of the individual equivalent concentrations in the blood, organs, and tissues over the maximum concentrations (CEQ_{max}) of female rats (Test 3) after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw

	Ratio [%] of the individual CEQ-values in the blood, organs and tissues over CEQ_{max}							
	Animal no.							
	276	277	278	279	280	281	282	283
	Time of sacrifice [hours after administration]							
Organ or tissue	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h
Blood	100.00	95.36	37.13	6.88	4.11	1.49	1.35	1.69
Liver	100.00	82.24	40.48	6.70	2.07	0.42	0.26	0.22
Renal cortex	100.00	78.71	30.21	4.12	2.18	n.c.	n.c.	n.c.
Renal medulla	87.38	100.00	30.97	4.91	2.39	n.c.	n.c.	n.c.
Kidney total	100.00	98.92	33.17	4.97	2.62	n.c.	n.c.	n.c.
Brown fat	100.00	47.17	28.48	2.36	0.86	n.c.	n.c.	n.c.
Perirenal fat	100.00	65.26	44.84	6.72	2.43	1.13	n.c.	n.c.
Skeleton muscle	100.00	28.70	15.32	1.39	n.c.	n.c.	n.c.	n.c.
Myocardium	100.00	52.61	28.34	2.87	0.80	n.c.	n.c.	n.c.
Lung	100.00	93.38	49.43	3.56	3.32	n.c.	n.c.	n.c.
Spleen	100.00	58.82	25.58	2.86	1.28	n.c.	n.c.	n.c.
Pancreas	100.00	55.23	24.34	n.c.	0.89	n.c.	n.c.	n.c.
Bone marrow	100.00	73.00	33.66	n.c.	n.c.	n.c.	n.c.	n.c.
Ovary	100.00	69.45	28.34	3.55	1.51	n.c.	n.c.	n.c.
Uterus	100.00	92.82	37.58	7.03	5.91	n.c.	n.c.	n.c.
Brain	100.00	31.41	20.01	1.59	n.c.	n.c.	n.c.	n.c.
Spinal cord	100.00	29.95	17.69	1.40	n.c.	n.c.	n.c.	n.c.
Pituitary gland	100.00	51.96	25.40	3.38	n.c.	n.c.	n.c.	n.c.
Pineal body	100.00	57.89	26.33	n.c.	n.c.	n.c.	n.c.	n.c.
Adrenal gland	100.00	62.83	29.39	3.38	1.47	0.64	n.c.	n.c.
Thymus	100.00	61.34	29.44	2.82	n.c.	n.c.	n.c.	n.c.
Thyroid gland	100.00	66.57	28.65	3.42	1.40	n.c.	n.c.	n.c.
Salivary gland	100.00	57.77	24.23	2.66	0.85	n.c.	n.c.	n.c.
Nasal mucosa	100.00	74.21	38.21	7.80	4.08	2.19	2.18	2.26
Vitreal body	34.78	100.00	20.85	8.35	n.c.	n.c.	n.c.	n.c.
Harderian gland	100.00	40.13	23.38	2.37	0.88	n.c.	n.c.	n.c.

$$\text{Ratio} = \frac{CEQ_{indiv.\ time}}{CEQ_{max}} \times 100\ [\%]$$

CEQ: Equivalent concentration [µg eq/g]
n.c.: not calculated because either TRR-values were < LOQ or organ/tissue was not discernible in the radioluminograms

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C. Excretion and exhalation of male and female rats (Test 2 and Test 3)

The major part of the radioactivity (up to 88% in male and up to 80% in female rats) was excreted with faeces and the minor part (up to 14% for both, male and female rats) with urine. 48 hours after administration more than 92% of the dose (male rats) or 87% of the dose (female rats) had been excreted and the excretion was nearly completed after 72 hours. Values up to 103.86% and 88.03% of total excretion of radioactivity had been observed for male and female rats, respectively.

The exhalation of $^{14}CO_2$ was tested with male and female animals for a period of 48 hours. Only a negligible amount of < 0.01% of the given dose was exhaled during this sampling period for both, male and female rats. This demonstrated that the ^{14}C labelling in the pyrazole-4 moiety of the molecule was stable with regard to formation of carbon dioxide in male and female rats.

Table 5.1.1- 36: Cumulative excretion of radioactivity in urine, faeces and expired air of male Rats (Test 2) after a single oral administration of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw

	Percent of radioactive dose administered (cumulative)							
	Animal no.							
	268	269	270	271	272	273	274	275
	Time of sacrifice [h after administration]							
	1	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Exhaled air								
24 h					0.0050	0.0069	0.0050	0.0046
48 h					0.0059	0.0079	0.0056	0.0053
Urine								
1 h	0.04							
4 h		3.44	2.27	3.18	1.57	4.65	4.39	1.32
8 h			6.23	5.04	2.63	7.39	8.15	6.55
24 h				9.22	6.29	12.32	13.21	11.39
48 h					6.62	12.91	13.56	11.74
72 h						12.99	13.60	11.82
96 h							13.64	11.86
120 h							13.66	11.90
144 h								11.92
168 h								11.93
Faeces								
24 h	*		*	82.21	72.83	74.86	75.04	80.02
48 h					85.62	89.73	83.44	87.58
72 h						90.86	84.21	88.06
96 h							84.30	88.15
120 h							84.35	88.20
144 h								88.24
168 h								88.27
Sum total	**0.04**	**3.44**	**6.23**	**91.43**	**92.25**	**103.86**	**98.02**	**100.20**

* faeces not collected

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Table 5.1.1- 37: Cumulative excretion of radioactivity in urine, faeces and expired air of female rats (Test 3) after a single oral administration of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw

	Percent of radioactive dose administered (cumulative)							
	Animal no.							
	276	277	278	279	280	281	282	283
	Time of sacrifice [h post administration]							
	1 h	**4 h**	**8 h**	**24 h**	**48 h**	**72 h**	**120 h**	**168 h**
Exhaled air								
24 h					0.0020	0.0011	0.0014	0.0016
48 h					0.0024	0.0012	0.0015	0.0019
Urine								
1 h	0.07							
4 h		3.32	0.07	0.52	2.98	1.50	0.27	0.25
8 h			2.68	2.02	3.26	1.96	2.69	2.38
24 h				6.80	11.49	7.10	6.89	6.98
48 h					13.66	7.71	7.84	8.20
72 h						7.84	7.94	8.31
96 h							7.96	8.33
120 h							7.98	8.34
144 h								8.35
168 h								8.35
Faeces								
24 h	*	*	*	61.83	47.50	67.59	56.16	60.24
48 h					74.27	79.03	75.44	78.69
72 h						80.22	76.63	80.01
96 h							76.77	80.18
120 h							76.80	80.20
144 h								80.25
168 h								80.28
Sum total	**0.07**	**3.32**	**2.68**	**68.63**	**87.94**	**88.06**	**84.78**	**88.63**

* faeces not collected

D. Metabolism (Test 1)

Radioactive residues in urine, faeces, plasma, organs and tissues

Total radioactivity was excreted up to 12.89% via urine and up to 81.02% via faeces of male rats within the 24 h experimental period. The total radioactivity in plasma samples decreased from 2.579 mg/kg at 1 h to 0.093 mg/kg at 24 h after dosing. A substantial reduction of the total radioactivity over time was observed for liver and kidney samples: The TRR in liver samples decreased from 11.787 mg eq/kg at 1 h after administration to 0.407 mg eq/kg at 24 h after administration. Total radioactivity in kidney samples decreased from 3.222 mg eq/kg at 1 h to 0.110 mg eq/kg at 24 h post administration. For the combined thyroid samples over all sampling points a value of 0.588 mg eq/kg was determined.

Efficiency of extraction

For metabolic investigation by HPLC analysis, faeces liver, kidney and thyroid samples were extracted with ACN/water (8/2; v/v). The extraction rates for faeces (0-8 h) amounted to 62.7% and for faeces (0-24 h) to 89.6%.

The extraction rates of organs ranged from 98.0% (1 h) to 46.3% (24 h) for liver extracts and from 98.5% (1 h) to 67.6% (24 h) for kidney extracts, decreasing with increasing time after administration. The extraction rate of the pooled thyroid samples was 97.5%.

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Metabolic profile in excreta

In urine samples up to 26 metabolites were detected. Parent compound could not be verified in urine. Three prominent metabolites could be identified in urine in this pilot metabolism study: BCS-CN88460-pyrazole-carboxylic acid, BCS-CN88460-cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1), and BCS-CN88460-cyclopropyl-pyrazole-carboxamide.

Parent compound was the largest fraction that was detected in the faeces extract (13.17% of dose) over a collection period up to 24 h. Beside parent compound up to 41 metabolites had been detected and 10 metabolites had been identified in the course of this study. The most prominent metabolites were BCS-CN88460-desmethyl-carboxylic acid (12.56%), BCS-CN88460-carboxylic acid (8.89%), BCS-CN88460-desmethyl-hydroxyphenyl-1,2-propandiol (6.75%), and BCS-CN88460-desmethyl-hydroxyphenyl-2-propanol (6.38%). Other metabolites identified were BCS-CN88460-desmethyl-lactic acid (4.08%), BCS-CN88460-desmethyl (2.82%), BCS-CN88460-lactic acid (1.48%), BCS-CN88460-desmethyl-hydroxymethyl-carboxylic acid (1.28%), BCS-CN88460-desmethyl-hydroxymethyl-diOH (1.01%), and BCS-CN88460-pyrazole-carboxylic acid (0.20%).

Metabolic profile in plasma

Parent compound was not detected in plasma samples. The most prominent component at 1 h post administration was the BCS-CN88460-desmethyl-carboxylic acid with values decreasing from 0.885 mg/kg at 1 h to 0.018 mg/kg at 8 h post administration. Other major metabolites in plasma samples were BCS-CN88460-cyclopropyl-pyrazole-carboxamide with the peak concentration at 4 h post administration (0.546 mg/kg) and BCS-CN88460-carboxylic acid (0.277 mg/kg; 1 h). Other metabolites identified and quantified were BCS-CN88460-desmethyl-lactic acid, BCS-CN88460-lactic acid, and BCS-CN88460-desmethyl.

Metabolic profile in liver

Overall, up to 35 metabolites were detected in liver samples beside the parent compound.

The most prominent components identified and quantified at 1 h after administration were BCS-CN88460-carboxylic acid (2.580 mg/kg), BCS-CN88460-desmethyl-carboxylic acid (1.749 mg/kg), and BCS-CN88460-lactic acid (0.895 mg/kg). BCS-CN88460-desmethyl lactic acid showed the highest concentration at 4 h after administration (1.144 mg/kg), decreasing to 0.014 mg/kg after 24 h.

Other metabolites identified and quantified were BCS-CN88460-desmethyl and BCS-CN88460-cyclopropyl-pyrazole-carboxamide with highest concentrations at 1 h post administration.

Parent compound could be quantified with 0.664 mg/kg at 1 h after administration decreasing to 0.068 mg/kg after 8 h. At 24 h post administration, no parent compound could be quantified in the liver sample.

Metabolic profile in kidney

Overall, up to 20 metabolites were detected in kidney samples beside the parent compound.

The most prominent components identified and quantified were BCS-CN88460-carboxylic acid (0.567 mg/kg), BCS-CN88460-desmethyl-carboxylic acid (0.539 mg/kg), and BCS-CN88460-cyclopropyl-pyrazole-carboxamide (0.237 mg/kg) with highest values at 1 h after administration.

Other metabolites identified and quantified were BCS-CN88460-cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1) (0.119 mg/kg), BCS-CN88460-desmethyl-lactic acid (0.115 mg/kg), BCS-CN88460-pyrazole-carboxylic acid (0.100 mg/kg), BCS-CN88460-desmethyl (0.097 mg/kg), and BCS-CN88460-lactic acid (0.045 mg/kg) with highest concentrations at 1 h post administration.

Parent compound could be quantified with 0.045 mg/kg at 1 h after administration decreasing to 0.068 mg/kg at 4 h. After 4 h post administration, no parent compound could be quantified in the kidney samples.

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Table 5.1.1- 38: Quantitative evaluation of parent compound and metabolites in urine after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw (Test 1)

	Urine	0 - 1 h	0 - 4 h	0 - 8 h	0 - 24 h
	% of dose	**0.52**	**3.23**	**5.47**	**12.89**
Peak ID	Report name: BCS-CN88460-	% of dose			
U1	unknown	---	---	0.01	0.58
U2	unknown	---	0.02	0.24	0.47
U3	unknown	---	0.06	0.24	0.48
U4	unknown	---	0.01	0.11	0.19
U5	pyrazole-carboxylic acid	0.06	0.27	0.27	0.33
U6	unknown	0.01	0.02	0.07	0.21
U7	cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1)	0.16	1.42	1.95	3.29
U8	unknown*	0.04	0.42	1.04	4.76
U9	unknown	0.01	0.03	0.18	0.31
U10	unknown	0.10	0.21	0.22	0.33
U11	unknown	0.01	0.04	0.09	0.19
U12	cyclopropyl-pyrazole-carboxamide	0.04	0.23	0.29	0.54
U13	unknown	0.03	0.07	0.10	0.18
U14	unknown	0.02	0.08	0.13	0.14
U15	unknown	---	0.03	0.06	0.11
U16	unknown	0.03	0.10	0.16	0.17
U17	unknown	---	0.07	0.11	0.16
U18	unknown	0.01	0.04	0.03	0.10
U19	unknown	---	0.01	0.02	0.04
U20	unknown	---	0.05	0.06	---
U21	unknown	---	---	---	0.12
U22	unknown	---	0.01	0.02	0.02
U23	desmethyl-lactic acid	---	0.03	0.02	0.07
U24	unknown	0.01	0.01	0.02	0.06
U25	unknown	---	---	0.01	0.02
U26	unknown	---	---	0.01	---
Sum total		**0.52**	**3.23**	**5.47**	**12.89**
Total identified		**0.26**	**1.94**	**2.54**	**4.23**
Total characterised		**0.26**	**1.29**	**2.93**	**8.66**

*) The metabolite was partly identified and therefore was reported as characterised, only. In addition the metabolite was not stable during storage as observed in the rat ADME study with the pyrazole label based on its profiling method.

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Table 5.1.1- 39: Quantitative evaluation of parent compound and metabolites in faeces after a single oral dose of 5 mg [pyrazole-4-14C]BCS-CN88460/kg bw (Test 1)

Peak ID	Faeces from animal (0 - 24 h) Report name: BCS-CN88460-	% of dose
F1	pyrazole-carboxylic acid	0.20
F2	unknown	0.15
F3	unknown	0.18
F4	unknown	0.30
F5	unknown	0.43
F6	unknown	0.73
F7	unknown	0.25
F8	unknown	0.24
F9	desmethyl-hydroxymethyl-diOH	1.01
F10	unknown	0.32
F11	desmethyl-hydroxyphenyl-1,2-propandiol	6.75
F12	unknown	0.27
F13	unknown	0.22
F14	unknown	0.63
F15	desmethyl-hydroxyphenyl-2-propanol	6.38
F16	unknown	1.36
F17	desmethyl-lactic acid	4.08
F18	desmethyl-hydroxymethyl-carboxylic acid	1.28
F19	unknown	1.24
F20	unknown	0.69
F21	lactic acid	1.48
F22	unknown	1.17
F23	unknown	0.26
F24	unknown	1.01
F25	unknown	0.18
F26	unknown	0.11
F27	desmethyl-carboxylic acid	12.56
F28	unknown	0.24
F29	unknown	0.99
F30	unknown	0.12
F31	carboxylic acid	8.89
F32	unknown	0.67
F33	unknown	0.37
F34	unknown	0.25
F35	unknown	0.26
F36	desmethyl	2.82
F37	parent compound	13.17
F38	unknown	0.25
F39	unknown	0.51
F40	unknown	0.21
F41	unknown	0.21
F42	unknown	0.16
Total identified		**58.61**
Total characterised		**13.99**
Solids (PLS)		8.41
Total balance		**81.02**

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Table 5.1.1- 40: Quantitative evaluation of metabolites in plasma after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw (Test 1)

	Plasma	1 h		4 h		8 h	
	TRR [mg/kg]	2.579		1.623		0.198	
Peak ID	Report name: BCS-CN88460-	% of TRR	[mg/kg]	% of TRR	[mg/kg]	% of TRR	[mg/kg]
P1	unknown	1.2	0.031	10.8	0.176	36.5	0.072
P2	unknown	0.7	0.018	---	---	---	---
P3	cyclopropyl-pyrazole-carboxamide	14.4	0.372	33.6	0.546	46.7	0.092
P4	unknown	0.8	0.021	---	---	---	---
P5	desmethyl-lactic acid	5.4	0.140	15.6	0.253	3.9	0.006
P6	unknown	7.8	0.202	2.0	0.032	---	---
P7	unknown	5.4	0.139	9.0	0.145	4.7	0.009
P8	lactic acid	1.1	0.027	---	---	---	---
P9	unknown	1.2	0.031	---	---	---	---
P10	unknown	1.5	0.038	---	---	---	---
P11	desmethyl-carboxylic acid	34.4	0.885	21.2	0.343	9.0	0.018
P12	unknown	5.4	0.139	2.8	0.045	---	---
P13	carboxylic acid	10.7	0.277	3.3	0.054	---	---
P14	unknown	0.8	0.022	---	---	---	---
P15	unknown	4.3	0.112	1.8	0.028	---	---
P16	unknown	0.5	0.013	---	---	---	---
P17	unknown	2.0	0.052	---	---	---	---
P18	desmethyl	2.3	0.060	---	---	---	---
Sum total		**100.0**	**2.579**	**100.0**	**1.623**	**100.0**	**0.198**
Total identified		**68.3**	**1.761**	**73.7**	**1.196**	**58.8**	**0.116**
Sum characterised		**31.7**	**0.818**	**26.3**	**0.427**	**41.2**	**0.082**

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Table 5.1.1- 41: Quantitative evaluation of parent compound and metabolites in liver after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw (Test 1)

	Liver	1 h		4 h		8 h		24 h	
	TRR [mg/kg]	11.787		4.764		2.496		0.407	
Peak ID	Report name: BCS-CN88460-	% of TRR	[mg/kg]	% of TRR	[mg/kg]	% of TRR	[mg/kg]	% of TRR	[mg/kg]
L1	unknown	---	---	---	---	2.7	0.068	3.5	0.014
L2	unknown	---	---	---	---	0.6	0.015	---	---
L3	unknown	1.6	0.185	2.9	0.138	4.2	0.105	2.9	0.012
L4	cyclopropyl-pyrazole-carboxamide	2.0	0.233	4.2	0.200	3.3	0.082	---	---
L5	unknown	0.7	0.085	---	---	0.5	0.014	---	---
L6	unknown	1.3	0.149	2.3	0.112	2.9	0.072	7.3	0.030
L7	unknown	---	---	---	---	0.5	0.012	---	---
L8	unknown	---	---	---	---	0.9	0.022	---	---
L9	unknown	---	---	---	---	0.6	0.016	---	---
L10	unknown	---	---	---	---	0.6	0.016	---	---
L11	unknown	---	---	---	---	1.7	0.042	---	---
L12	unknown	---	---	---	---	1.1	0.028	1.8	0.008
L13	unknown	---	---	---	---	0.4	0.010	---	---
L14	unknown	---	---	---	---	0.4	0.011	---	---
L15	unknown	---	---	2.4	0.114	1.1	0.026	---	---
L16	desmethyl lactic acid	5.4	0.632	24.0	1.144	6.2	0.154	3.3	0.014
L17	unknown	8.0	0.945	2.9	0.137	3.5	0.088	---	---
L18	unknown	0.8	0.099	---	---	1.4	0.034	---	---
L19	lactic acid	7.6	0.895	14.1	0.670	12.8	0.320	2.1	0.009
L20	unknown	---	---	---	---	0.8	0.020	---	---
L21	unknown	3.0	0.349	---	---	1.1	0.027	---	---
L22	unknown	---	---	---	---	1.2	0.031	---	---
L23	unknown	2.7	0.314	---	---	2.4	0.061	---	---
L24	unknown	2.7	0.316	3.6	0.172	4.7	0.117	8.1	0.033
L25	unknown	0.5	0.062	---	---	0.7	0.019	3.0	0.012
L26	desmethyl-carboxylic acid	14.8	1.749	15.2	0.726	7.2	0.180	7.3	0.030
L27	unknown	7.8	0.922	5.1	0.244	3.8	0.096	---	---
L28	carboxylic acid	21.9	2.580	9.6	0.459	8.3	0.206	7.0	0.028
L29	unknown	2.6	0.312	---	---	0.8	0.020	---	---
L30	unknown	3.3	0.389	---	---	1.5	0.038	---	---
L31	unknown	---	---	---	---	0.5	0.013	---	---
L32	unknown	---	---	---	---	0.3	0.008	---	---
L33	unknown	---	---	---	---	0.4	0.009	---	---
L34	desmethyl	5.2	0.612	2.5	0.121	2.4	0.059	---	---
L35	unknown	0.5	0.058	---	---	1.0	0.025	---	---
L36	parent compound	5.6	0.664	2.4	0.116	2.7	0.068	---	---
Total identified		**62.5**	**7.364**	**74.5**	**3.551**	**42.2**	**1.052**	**19.7**	**0.080**
Total characterised		**35.5**	**4.186**	**16.8**	**0.802**	**40.4**	**1.009**	**26.6**	**0.108**
Solids (PLS)		2.0	0.238	8.6	0.411	17.4	0.435	53.7	0.218
Total balance		**100.0**	**11.787**	**100.0**	**4.764**	**100.0**	**2.496**	**100.0**	**0.407**

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Table 5.1.1- 42: Quantitative evaluation of parent compound and metabolites in kidney after a single oral dose of 5 mg [pyrazole-4-^{14}C]BCS-CN88460/kg bw (Test 1)

	Kidney	1 h		4 h		8 h		24 h	
	TRR [mg eq/kg] *)	3.222		0.896		0.379		0.110	
Peak ID	Report name: BCS-CN88460-	% of TRR	[mg/kg]	% of TRR	[mg/kg]	% of TRR	[mg/kg]	% of TRR	[mg/kg]
K1	unknown	---	---	2.9	0.026	3.9	0.015	---	---
K2	pyrazole-carboxylic acid	3.1	0.100	---	---	3.1	0.012	---	---
K3	cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1)	3.7	0.119	5.0	0.044	3.5	0.013	3.4	0.004
K4	unknown	1.4	0.046	18.7	0.163	43.9	0.167	48.1	0.053
K5	unknown	0.8	0.025	---	---	---	---	---	---
K6	cyclopropyl-pyrazole-carboxamide	7.4	0.237	19.8	0.178	17.7	0.067	16.2	0.018
K7	unknown	1.9	0.060	---	---	---	---	---	---
K8	unknown	1.4	0.046	---	---	---	---	---	---
K9	unknown	0.8	0.026	---	---	---	---	---	---
K10	desmethyl-lactic acid	3.6	0.115	9.8	0.087	---	---	---	---
K11	unknown	11.8	0.382	5.2	0.046	3.6	0.014	---	---
K12	lactic acid	1.4	0.045	---	---	---	---	---	---
K13	unknown	1.8	0.058	---	---	---	---	---	---
K14	desmethyl-carboxylic acid	16.7	0.539	12.8	0.115	5.8	0.022	---	---
K15	unknown	5.2	0.168	4.4	0.039	2.9	0.011	---	---
K16	carboxylic acid	17.6	0.567	6.7	0.060	3.8	0.015	---	---
K17	unknown	1.1	0.035	---	---	---	---	---	---
K18	unknown	6.4	0.206	2.9	0.026	3.3	0.012	---	---
K19	unknown	0.8	0.026	2.4	0.022	---	---	---	---
K20	desmethyl	3.0	0.097	2.1	0.019	---	---	---	---
K21	parent compound	1.4	0.045	2.4	0.021	---	---	---	---
Total identified		**97.0**	**2.942**	**58.5**	**0.525**	**34.0**	**0.129**	**19.5**	**0.022**
Total characterised		**33.4**	**1.077**	**36.1**	**0.323**	**57.6**	**0.219**	**48.1**	**0.053**
Solids (PES)		1.5	0.049	5.1	0.046	8.4	0.032	32.4	0.036
Total balance		**100.0**	**3.222**	**100.0**	**0.896**	**100.0**	**0.379**	**100.0**	**0.110**

III. CONCLUSIONS

The insecticide BCS-CN88460, labelled with ^{14}C in the pyrazole-4 moiety of the molecule, was obviously absorbed very fast from the gastrointestinal tract of male and female Wistar rats after single oral administration leading to maximum plasma levels in the systemic compartment blood already 1 h after dosing. Most of the radioactivity was excreted via the faeces and only a minor part with urine. The excretion was nearly completed after 3 days.

The absorbed test compound related radioactivity was distributed throughout the animal bodies immediately after dosing with a clear preference to the liver and kidney that are the responsible organs for metabolism (liver) and excretion (kidney). In case of females additionally a preference to the myocardium, some glandular organs (e.g. harderian gland), and fatty tissues was observed.

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Maximum concentrations of radioactivity were reached 1 h after dosing in most organs and tissues of both sexes. The compound related radioactivity was rapidly and almost completely eliminated from the central and peripheral compartments of the animals at the time of sacrifice. At that time, trace amounts of radioactivity were detected only in blood, liver, kidney, lung, adrenal gland and nasal mucosa of male rats as well as in blood, liver, and nasal mucosa of female rats. This shows that the residual radioactivity is excreted predominantly by the faecal route smoothly from the animal bodies.

No relevant sex related differences concerning the maximum equivalent concentrations in blood, organs, and tissues were observed between male and female rats. Excretion was slightly faster in male rats.

The results of the WBA indicated that male and female rats exhibited a very similar absorption, distribution and excretion behaviour. Any accumulation or substantial retention of [pyrazole-4-^{14}C]BCS-CN88460 related residues in organs and tissues of male and female rats can be excluded.

The results of the pilot metabolism experiment with male rats confirmed basically the observations made in the WBA referring to the absorption and excretion.

More than 90% of the total radioactivity was excreted within 24 h after dosing. The excretion was predominantly faecal.
Approximately 13% of the unchanged parent compound had been excreted via faeces. In urine samples parent compound was not identified.

The most important metabolic reactions of [pyrazole-4-^{14}C]BCS-CN88460 were the demethylation of the pyrazole moiety and the hydroxylation in position 1 and 2 of the propyl group, in the phenyl ring, and after defluorination of the difluoromethyl moiety was leading to mono-, di-, or tri-hydroxy compounds. Hydroxylation at other positions was also detected, but was not exactly located by structure elucidation.
Another important metabolic reaction was the carboxylation of the 1-propanol group leading to a carboxylic acid or in combination with a 2-propanol group to a lactic acid group.
Cleavage was detected for the phenyl moiety and was leading to a carboxamide. Cleavage of the cyclopropyl ring in combination with cleavage of the phenyl moiety was leading to BCS-CN88460-pyrazole-carboxylic acid.

In addition, the conjugation with glucuronic acid of BCS-CN88460-cyclopropyl-pyrazole-carboxamide was observed.

From these pilot metabolism investigations the following metabolite pathway can be depicted:

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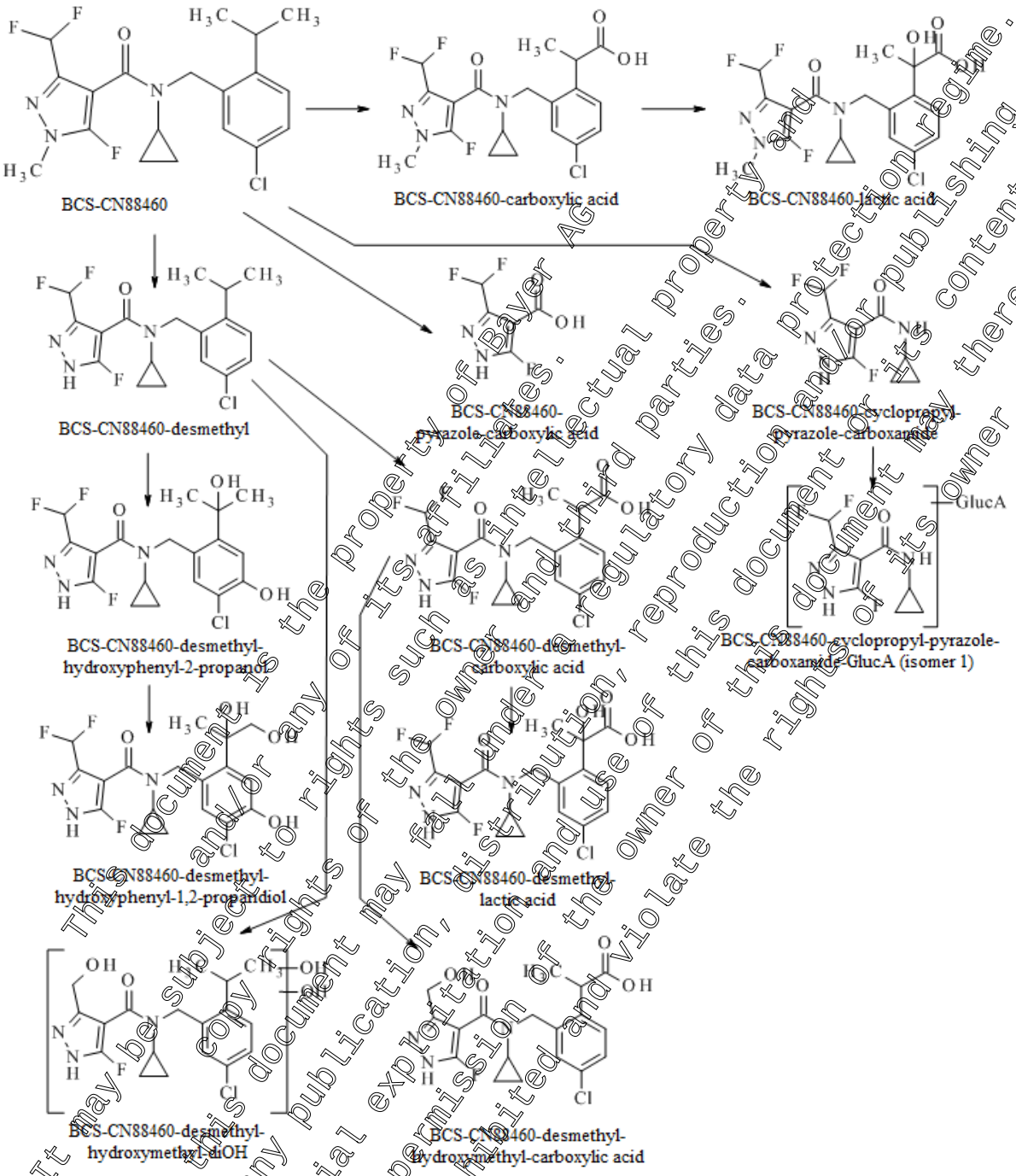
Figure 5.1.1- 3: Proposed metabolic pathway of [pyrazole-4-^{14}C]BCS-CN88460 in the rat



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Report:	KCA 5.1.1/04; [redacted]; [redacted]; 2017; M-590199-02-1
Title:	Amendment 1 to [phenyl-UL-14C]BCS-CN88460: Tissue distribution and excretion of radioactivity in the rat by quantitative whole body autoradiography - Amended final report 1
Report No.:	EnSa-16-1023
Document No.:	M-590199-02-1
Guideline(s):	OECD Guideline for Testing Chemicals, 417 US EPA OCSPP 870.7485 Japanese MAFF Test Guideline 12 Nousan 8147
Guideline deviation(s):	none
GLP/GEP:	**yes**

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Executive Summary

This study was conducted in order to determine the tissue distribution of radioactivity in the male and female albino rat following a single oral administration of [phenyl-UL-^{14}C]BCS-CN88460, at a nominal dose level of 5 mg BCS-CN88460/kg body weight, using quantitative whole-body autoradiography techniques. Pharmacokinetic parameters of radioactivity in tissues and plasma were also determined.

Following dose administration all animals were placed in glass metabolism cages suitable for the separate collection of urine and faeces. At the scheduled sampling times, animals were deeply anaesthetised under isoflurane and killed by immersion in a freezing mixture of hexane and solid carbon dioxide.

For animals scheduled for sacrifice at 168 hours, 200 µL of blood was collected from the tail vein at 15 minutes, 1, 2, 7, 24, 48, 72, 96, 120, 144 and 168 hours post-dose.

Radioactivity was widely distributed following oral administration. Radioactivity concentrations were measured in plasma using liquid scintillation counting methods. Once fully frozen, the carcasses were subjected to whole-body autoradiography procedures. Radioactivity concentrations in tissues were quantified from the whole-body autoradiograms, using a validated image analysis system.

Following oral administration of [phenyl-UL-^{14}C]BCS-CN88460 to male and female albino rats, radioactivity was absorbed and distributed. Peak concentrations were generally attained at the first sampling time, 1 hour.

Radioactivity was rapidly eliminated from tissues, so that by the final sampling time of 168 hours in albino rats, the majority of tissues (100% and 94% in male and female animals, respectively) were devoid of quantifiable levels of radioactivity. Elimination half-lives for radioactivity were typically <20 hours in male animals and generally <10 hours in female animals. Longer elimination half-lives (>20 hours) in male animals were observed in liver, brown fat, urinary bladder wall and lung. Tissues in female animals with half-lives >10 hours were the liver, bone marrow and plasma.

Tissue: blood concentration ratios were less than one in over 50% of the measured tissues over the course of the study. Tissues in which the ratio was consistently greater than one were the bile ducts, liver, renal cortex, renal medulla, urinary bladder (wall and contents) and regions of the gastrointestinal tract.

Highest concentrations of radioactivity were found in the liver and bile ducts and other tissues typically associated with excretion (namely the kidney, contents and mucosa/wall of the urinary bladder and oesophageal gastrointestinal tract) of male and female animals. This indicated that elimination of radioactivity was via the gastrointestinal tract and the renal system. Results from the analysed excreta indicated that faecal elimination was the principal route of elimination.

Tissue concentrations were typically below 1.0 µg equiv/g after oral administration. Higher radioactivity concentrations (excluding tissues of the gastrointestinal tract), were determined in the bile ducts, liver, kidney (cortex and medulla) and bulbo-urethral gland (males). Highest total

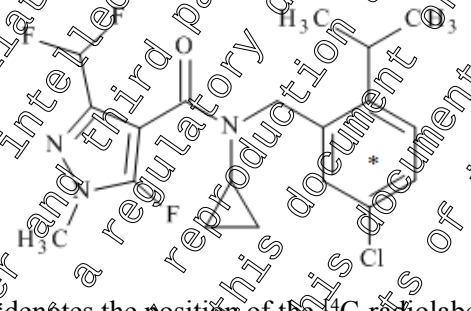
exposures ($AUC_{0\text{-last}}$ of above 20 µg equiv.h/g, to a maximum of 102 µg equiv.h/g) in male animals were observed in bile ducts, preputial gland, liver and various regions of the gastrointestinal tract. In female tissues exposures ($AUC_{0\text{-last}}$ of greater than 20 µg equiv.h, to a maximum of 166 µg equiv.h) were observed in bile ducts, liver and clitoris.

Low concentrations of radioactivity were measured in the tissues of the central nervous system, *ie* the brain and spinal cord, which declined over the duration of the study. Other tissues which generally contained low levels of radioactivity were the bone surface and lens of the eye.

I. Material and Methods

A. Material

1. Test material:

Test substance	
Chemical structure	 *denotes the position of the ^{14}C-radiolabel
Radiolabelled test material	[Phenyl-UL-^{14}C]BCS-CN88460
Specific radioactivity	4.13 MBq/mg = 2.48 x 10^8 dpm/mg
Chemical purity	>98% (HPLC)
Radiochemical purity	>98% (HPLC)
Vehicle	0.5% aqueous Tragacanth solution
Preparation of dosing solution	radiolabelled test compound suspended in aqueous Tragacanth solution

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2. Test animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	HsDCPB: WU (Wistra Unilever)
Breeding facility:	[redacted]
Sex and numbers involved:	Males: 8 animals; Female: 8 animals
Body weight:	Male animals: 226 - 244 g at the time of administration. Female animals: 176 - 191 g at the time of administration.
Acclimatization:	During the acclimation periods, the animals were kept in suitable solid floor cages, containing suitable bedding provided with wooden Aspen chew blocks and polycarbonate tunnels for about 5 days prior to the administration
Identification:	Rats were uniquely numbered by tail marking. Numbers were allocated randomly. Cages were coded by cards giving information including study number and animal number. The study room was identified by a card giving information including room number and study number.
Housing:	During the testing periods, the rats were kept individually in glass metabolism cages. With these cages, an almost quantitative and separate collection of urine and faeces was possible. While in cages, the animals had unrestricted access to food and water. Temperature 20 - 24 °C, relative humidity 34 - 65%; 12 / 12 hours light / dark cycle; 15 to 20 air changes/hour.
Feed and water:	The rats were allowed ad libitum access to 5LE2 EU Rodent Diet 14%. The diet supplier provided an analysis of the concentration of certain contaminants and some nutrients for each batch used.

B. Study design

1. Dosing

Each animal received a single oral administration by gavage at a nominal dose volume of 10 mL/kg. Nominal doses of 5 mg/kg body weight were administered to each animal and the radioactive doses were ca 20.2 MBq/kg body weight. Due to different animal weights at administration, the mean actual dose in the tests amounted to 4.86 - 4.90 mg/kg body weight or to 4.87 - 4.95 mg/kg body weight for male and female rats, respectively. The stability of the test compound in the suspensions was assured by radio-HPLC analysis after administration.

2. Sampling

Following dose administration all animals were placed in glass metabolism cages suitable for the separate collection of urine and faces. Collection vessels for urine and faeces were cooled with solid carbon dioxide.

Urine was collected separately for each animal in intervals of 1h, 4 h, 8 h, 24 h and every 24 h until 168 h after dose administration. Urine collection vessels were rinsed with a small volume (2 to 5 mL) of water and this was added to the urine sample. The volume was measured and radioactivity was determined by liquid scintillation counting methods.

The faeces samples were collected every 24 h until 168 h separately for each animal.

At each collection of excreta, cage debris was removed and the cages rinsed with a small volume of water. After the final water wash, methanol was used to wash the cages and added to the final water wash. The volume was measured and radioactivity was determined by liquid scintillation counting methods.

Cage debris was pooled separately for each animal over the collection period.

Faeces and cage debris were homogenised in an appropriate volume of deionised water. A suitable volume of solubilising agent was added to the samples and radioactivity was determined by liquid scintillation counting methods.

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Urine and faeces samples were stored at <-50°C and cage debris, cage washings and carcasses were stored at <-10°C.

3. Sacrifice
One animal of each sex was removed from its metabolism cage 1, 4, 8, 24, 48, 72, 120 and 168 hours after dose administration. At the scheduled sampling times, animals were deeply anaesthetised under isoflurane and killed by immersion in a freezing mixture of hexane and solid carbon dioxide for at least 20 minutes.

4. Preparation of plasma samples
For animals scheduled for sacrifice at 168 hours, 200 µL of blood was collected from the tail vein at 15 minutes, 1, 2, 7, 24, 48, 72, 96, 120, 144 and 168 hours post-dose.
The samples were centrifuged (10 minutes, 3000 rpm, 4°C) in order to obtain plasma and the radioactivity concentration was measured using liquid scintillation counting methods.

5. Quantitative whole-body autoradiography
The distribution of total radioactivity without differentiation of unchanged test compound and radiolabelled biotransformation products in, and the elimination from blood, organs, and tissues was determined qualitatively and quantitatively at various time points by WBAL.
For WBAL the sacrificed animals were freeze-embedded in a mould containing 2% (w/v) aqueous CMC (carboxymethylcellulose) paste. A number of longitudinal, sagittal sections (nominally 30 µm) were taken, normally at each of 5 levels from each rat carcass. Coordinates for sectioning included, but were not limited to the following tissues: ovary, intra-orbital lachrymal gland, Harderian gland, adrenal gland, thyroid, brain and spinal cord.

Apposition autoradiograms (radioluminograms) were obtained on Fuji Imaging Plates (type BAS-MS) using a Fuji radioluminography system. All sections were exposed to imaging plates for a minimum of 7 days and enlargements from autoradiograms of areas of interest were presented, if appropriate.
Carbon-14 blood standards (pre-prepared at Covance) were included with each autoradiogram. Whole-body autoradiograms were analysed using a validated PC-based image analysis package (Seescan2 software, Labl ogic Systems Ltd). The blood standards included with each autoradiogram were used to construct a calibration curve over a range of radioactivity concentrations. The sites of tissue accumulation of radioactivity was identified by visual inspection of the autoradiograms and quantified by reference to the calibration curves.

6. Tissue half-life and AUC determination
Tissue half-life and AUC calculations for [phenyl-UL-^{14}C]BCS-CN88460 in plasma, blood and tissues were performed using the validated non-compartmental pharmacokinetic analysis programme Phoenix version 6.4 (Pharsight Corporation). All computations utilised the nominal sampling times recorded during the study. Plasma, blood and tissue concentrations that were below the limit of detection were assigned a value of zero for the purposes of tissue half-life and AUC determination. Samples marked as N8 (tissue not sectioned) were omitted from the analysis. For generated values to be considered reliable the coefficient of determination (Rsq value) had to be greater than 0.8 and 3 or more sampling concentrations had to be measured during the elimination phase.

- AUC_{0-last} - area under the plasma concentration-time curve calculated from 0-last, where last is the last measureable concentration, was calculated by non-compartmental analysis using the log/linear trapezoidal rule.
- $AUC_{0-\infty}$ - area under the plasma concentration-time curve extrapolated to infinite time, was calculated by non-compartmental analysis using the log/linear trapezoidal rule.
- C_{max} - maximum plasma concentration following the each dose was determined from observed values of the concentration-time profile.
- T_{max} - time of maximum concentration profile was determined by visual inspection of the concentration-time profile.

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- Elimination half life (t½) for plasma was determined by linear regression of at least three data points on the log (concentration) vs time plot, with a correlation co-efficient (R2) of 0.8 or greater (this was subject to sufficient data points being available to define the terminal portion of the curve.

II. RESULTS AND DISCUSSION

A. Distribution of radioactivity in male rats

Peak concentrations of radioactivity in the tissues of male albino rats typically occurred at the first sampling time of 1 hour. The only exceptions being the blood, nasal mucosa, pineal body, prostate and trachea, where peak concentrations were observed 4 hours after dosing, and the preputial gland where then peak concentration was determined at 24 hours post-dose.

All investigated tissues were exposed to drug-related radioactivity at one or more of the sampling times. Radioactivity concentrations declined over the duration of the study so that at the penultimate sampling time (120 hours) only 13% of the investigated tissues including the preputial gland, liver, nasal mucosa and blood retained quantifiable levels of radioactivity. At the final sampling time of 168 hours, radioactivity was not quantifiable in any tissues.

Concentrations of drug-related radioactivity in blood were 0.460, 0.464, 0.197, 0.0896, 0.0229 and 0.0318 µg equiv/g at 1, 4, 8, 24, 48 and 72 hours after dosing, respectively.

Highest concentrations of radioactivity (>1.0 µg equiv/g to a maximum of 16.1 µg equiv/g at one or more sampling time) were observed in the bile ducts, bulbo-urethral gland, liver, preputial gland, renal cortex and renal medulla. Lowest concentrations, at each sampling time, were generally associated with the bone surface, brain, choroid plexus, lens of the eye, meninges, muscle, nasal mucosa, peri-renal fat, seminal vesicles, spinal cord, testis and white fat.

The elimination half-lives of radioactivity from tissues, where reliable measurements could be ascertained, were generally less than 20 hours. The only exceptions, (half-lives >20 hours), were observed in liver (53.6 hours), brown fat (26.2 hours), and lung (23.4 hours).

$AUC_{0\text{-}last}$ values were typically below 20 µg equiv.h/g for the majority of tissues. Tissues exhibiting higher exposures ($AUC_{0\text{-}last}$) to drug-related radioactivity were the bile ducts (102 µg equiv.h/g), preputial gland (75.1 µg equiv.h/g), liver (55.6 µg equiv.h/g) and various regions of the gastrointestinal tract mucosa (37.0 to 27.4 µg equiv.h/g). $AUC_{0\text{-}last}$ values of ≤1.00 µg equiv.h/g were determined in the spleen, skin (non-pigmented), muscle, testis, meninges, thymus, peri-renal fat, white fat, spinal cord, brain and bone surface.

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Table 5.1.1- 43: Distribution of radioactivity in organs and tissues of male rats after a single oral dose of 5 mg [phenyl-UL-^{14}C]BCS-CN88460/kg bw

	µg equivalents of BCS-CN88460/g of tissue							
Animal Number and Sex	**231M**	**230M**	**225M**	**226M**	**227M**	**228M**	**229M**	**232M**
Tissue Sampling time (hours)	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Blood	0.460	0.464	0.197	0.0896	0.0229	0.0318	BLQ	BLQ
Adrenal cortex	0.586	0.312	0.142	0.0343	BLQ	BLQ	BLQ	BLQ
Adrenal medulla	0.666	0.379	0.147	0.0500	0.0233	BLQ	BLQ	BLQ
Aortic wall	0.329	0.299	0.138	0.0456	BLQ	BLQ	BLQ	BLQ
Bile ducts	5.25	3.42	2.17	2.59	0.520	0.104	ND	ND
Bone marrow	0.242	0.143	0.0686	0.0307	BLQ	BLQ	ND	ND
Bone surface	0.0235	0.0198	BLQ	BLQ	BLQ	ND	ND	ND
Brain	0.124	0.0525	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Brown fat	0.707	0.385	0.129	0.0375	BLQ	0.0188	BLQ	BLQ
Bulbo-urethral gland	1.84	0.240	0.157	0.0432	BLQ	BLQ	BLQ	BLQ
Caecum mucosa	0.485	7.39*	0.432*	0.525*	0.0872*	0.0224*	BLQ	BLQ
Choroid plexus	0.278	0.245	0.0342	BLQ	BLQ	BLQ	BLQ	ND
Epididymis	0.184	0.154	0.165	0.0195	BLQ	BLQ	ND	ND
Exorbital lachrymal gland	0.568	0.240	0.140	0.0285	BLQ	BLQ	BLQ	ND
Harderian gland	0.438	0.240	0.0722	BLQ	BLQ	BLQ	BLQ	ND
Intra-orbital lachrymal gland	0.399	0.238	0.130	0.0385	BLQ	BLQ	BLQ	ND
Large intestine mucosa	0.295	0.288	0.286	0.803	0.345*	0.0327	BLQ	BLQ
Lens	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	ND
Liver	3.72	3.52	1.63	0.670	0.157	0.114	0.0618	BLQ
Lung	0.530	0.436	0.166	0.0726	BLQ	0.0226	BLQ	BLQ
Mandibular lymph nodes	0.344	0.205	0.0726	BLQ	BLQ	BLQ	BLQ	ND
Meninges	0.135	0.0985	0.0876	BLQ	BLQ	ND	BLQ	BLQ
Muscle	0.197	0.101	0.0496	BLQ	BLQ	BLQ	BLQ	BLQ
Myocardium	0.533	0.350	0.116	0.0408	BLQ	BLQ	BLQ	BLQ
Nasal mucosa	0.181	0.268	0.113	0.158	0.0400	0.0222	0.0429	BLQ
Oesophageal wall	0.269	0.254	0.122	BLQ	BLQ	ND	ND	ND
Pancreas	0.742	0.240	0.0997	0.0242	BLQ	BLQ	BLQ	BLQ
Periodontal membrane	0.246	0.168	0.0601	0.0202	BLQ	BLQ	BLQ	ND
Peri-renal fat	0.134	0.0831	0.0301	BLQ	BLQ	BLQ	BLQ	BLQ
Pineal body	0.269	0.279	0.101	BLQ	BLQ	BLQ	BLQ	ND
Pituitary gland	0.232	0.146	0.0517	BLQ	BLQ	ND	ND	ND
Preputial gland	0.152	0.517	0.682	1.51	0.570	0.220	0.831	BLQ
Prostate	0.292	0.330	0.0791	0.0223	BLQ	BLQ	BLQ	BLQ
Rectum mucosa	0.193	0.113	0.0803	0.0269	0.0267	BLQ	0.0199	BLQ
Renal cortex	1.13	0.747	0.416	0.151	0.0419	0.0376	BLQ	BLQ
Renal medulla	1.05	0.366	0.255	0.126	0.0263	BLQ	BLQ	BLQ
Salivary glands	0.420	0.208	0.0593	0.0241	BLQ	BLQ	BLQ	ND
Seminal vesicles	0.247	0.0408	0.0771	0.141	BLQ	BLQ	BLQ	BLQ

Upper limit of quantification: 27.5 µg equiv/g for all measurements
Lower limit of quantification: 0.0183 µg equiv/g for all measurements
BLQ - Concentration below the lower limit of quantification
ND – Radioactivity not detected
*Tissues may be affected by high levels of radioactivity in the associated contents

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Table 5.1.1- 43 continued

	µg equivalents of BCS-CN88460/g of tissue							
Animal Number and Sex	**231M**	**230M**	**225M**	**226M**	**227M**	**228M**	**229M**	**232M**
Tissue **Sampling time (hours)**	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Skin (Non-pigmented)	0.225	0.122	0.0658	BLQ	BLQ	BLQ	BLQ	BLQ
Small intestine mucosa	8.51*	1.78*	0.296*	0.113	0.0376	0.0495	BLQ	BLQ
Spinal cord	0.137	0.0513	BLQ	BLQ	BLQ	BLQ	ND	ND
Spleen	0.408	0.152	0.069	BLQ	NS	BLQ	BLQ	BLQ
Stomach mucosa (fundus)	0.593	0.706	0.121	0.126	BLQ	BLQ	BLQ	BLQ
Stomach mucosa (non-fundic)	14.9*	1.12	0.843	0.0896	BLQ	0.0206	BLQ	BLQ
Testis	0.152	0.113	0.0674	BLQ	BLQ	BLQ	BLQ	BLQ
Thymus	0.201	0.0997	0.0255	BLQ	BLQ	BLQ	BLQ	BLQ
Thyroid	0.348	0.215	0.0771	BLQ	BLQ	BLQ	ND	ND
Tongue	0.468	0.237	0.0847	0.0226	BLQ	BLQ	BLQ	ND
Tooth pulp	0.412	0.392	0.101	0.0372	BLQ	BLQ	BLQ	ND
Trachea	0.184*	0.371	0.0448	BLQ	BLQ	ND	ND	ND
Urinary bladder wall	2.84	16.1	1.38	0.944	0.305	0.103	0.0871	BLQ
Urine	2.31*	13.5	2.46	0.985	0.194	0.0796	0.0899	BLQ
Uveal tract/retina	0.229	0.184	0.108	0.032	BLQ	BLQ	BLQ	ND
White fat	0.117	0.102	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ

Upper limit of quantification: 27.3 µg equiv/g for all measurements
Lower limit of quantification: 0.0183 µg equiv/g for all measurements
BLQ - Concentration below the lower limit of quantification
ND – Radioactivity not detected
*Tissues may be affected by high levels of radioactivity in the associated contents

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Table 5.1.1- 44: Tissue : blood ratios of radioactivity in the tissues of male rats after a single oral dose of 5 mg [phenyl-UL-^{14}C]BCS-CN88460/kg bw

	µg equivalents of BCS-CN88460/g of tissue							
Animal Number and Sex	**231M**	**230M**	**225M**	**226M**	**227M**	**228M**	**229M**	**232M**
Tissue Sampling time (hours)	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Blood	1.00	1.00	1.00	1.00	1.00	1.00	NC	NC
Adrenal cortex	1.27	0.671	0.721	0.383	0.739	0.542	NC	NC
Adrenal medulla	1.45	0.816	0.748	0.559	1.02	0.436	NC	NC
Aortic wall	0.715	0.645	0.699	0.509	0.541	0.383	NC	NC
Bile ducts	11.4	7.37	11.0	28.9	22.7	3.28	NC	NC
Bone marrow	0.525	0.308	0.548	0.342	0.376	0.259	NC	NC
Bone surface	0.0511	0.0427	NC	0.0676	0.0791	NC	NC	NC
Brain	0.269	0.113	NC	NC	0.00479	0.168	NC	NC
Brown fat	1.54	0.830	0.654	0.419	0.731	0.591	NC	NC
Bulbo-urethral gland	4.00	0.617	0.797	0.482	0.726	0.374	NC	NC
Caecum mucosa	1.06	15.98*	2.19*	5.86*	3.82*	0.703*	NC	NC
Choroid plexus	0.605	0.528	0.174	0.0541	0.396	0.272	NC	NC
Epididymis	0.399	0.332	0.840	0.218	0.449	0.415	NC	NC
Exorbital lachrymal gland	1.23	0.517	0.711	0.318	0.531	0.0311	NC	NC
Harderian gland	0.952	0.517	0.367	0.195	0.148	0.338	NC	NC
Intra-orbital lachrymal gland	0.868	0.512	0.658	0.429	0.286	0.426	NC	NC
Large intestine mucosa	0.641	0.621	4.45	8.96	15.1*	1.03	NC	NC
Lens	NC	0.0355	NC	NC	NC	0.162	NC	NC
Liver	8.08	7.599	8.26	7.48	6.88	3.58	NC	NC
Lung	1.15	0.939	0.844	0.811	0.772	0.711	NC	NC
Mandibular lymph nodes	0.683	0.442	0.369	0.0406	0.213	0.308	NC	NC
Meninges	0.294	0.212	0.445	0.163	NC	NC	NC	NC
Muscle	0.428	0.217	0.252	0.122	0.245	0.186	NC	NC
Myocardium	1.16	0.754	0.588	0.455	0.287	0.543	NC	NC
Nasal mucosa	0.394	0.577	0.572	1.73	1.75	0.697	NC	NC
Oesophageal wall	0.584	0.547	0.619	0.0757	0.315	NC	NC	NC
Pancreas	1.61	0.517	0.506	0.270	0.695	0.353	NC	NC
Periodontal membrane	0.534	0.363	0.305	0.226	0.0719	0.120	NC	NC
Peri-renal fat	0.292	0.179	0.163	0.169	0.247	0.233	NC	NC
Pineal body	0.585	0.601	0.514	NC	0.187	0.307	NC	NC
Pituitary gland	0.519	0.314	0.262	0.154	0.176	NC	NC	NC
Preputial gland	0.331	1.11	3.46	16.8	24.9	6.90	NC	NC
Prostate	0.635	0.730	0.402	0.249	0.479	0.205	NC	NC
Rectum mucosa	0.419	0.243	0.408	0.300	1.17	0.279	NC	NC
Renal cortex	2.46	1.61	2.11	1.69	1.83	1.18	NC	NC
Renal medulla	2.29	0.790	1.30	1.40	1.15	0.504	NC	NC
Salivary glands	0.912	0.449	0.301	0.269	0.129	0.273	NC	NC
Seminal vesicles	0.536	0.0878	0.391	1.58	0.330	0.198	NC	NC

Upper limit of quantification: 27.5 µg equiv/g for all measurements
Lower limit of quantification: 0.0183 µg equiv/g for all measurements

NC – Not calculated

*Tissues may be affected by high levels of radioactivity in the associated contents

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Table 5.1.1- 44 continued

	µg equivalents of BCS-CN88460/g of tissue							
Animal Number and Sex	**231M**	**230M**	**225M**	**226M**	**227M**	**228M**	**229M**	**232M**
Tissue Sampling time (hours)	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Skin (Non-pigmented)	0.490	0.263	0.334	0.168	0.276	0.306	NC	NC
Small intestine mucosa	18.5*	3.84*	1.50*	1.27	1.65	1.56	NC	NC
Spinal cord	0.297	0.110	0.0209	NC	0.032	0.200	NC	NC
Spleen	0.886	0.327	0.350	0.157	NC	0.309	NC	NC
Stomach mucosa (fundus)	1.29	1.52	0.615	1.40	0.680	0.367	NC	NC
Stomach mucosa (non-fundic)	32.5	2.42	4.28	1.00	0.659	0.646	NC	NC
Testis	0.331	0.243	0.342	0.107	0.556	0.204	NC	NC
Thymus	0.438	0.215	0.130	NC	0.033	0.177	NC	NC
Thyroid	0.756	0.464	0.391	0.186	0.407	0.276	NC	NC
Tongue	1.02	0.511	0.430	0.252	0.386	0.330	NC	NC
Tooth pulp	0.895	0.844	0.514	0.415	NC	0.252	NC	NC
Trachea	0.399	0.800	0.227	NC	0.170	NC	NC	NC
Urinary bladder wall	6.17	34.8	7.01	10.5	13.3	3.23	NC	NC
Urine	5.03*	29.1	12.5	11.0	8.48	2.50	NC	NC
Uveal tract/retina	0.498	0.396	0.549	0.357	0.340	0.372	NC	NC
White fat	0.254	0.219	0.0811	NC	0.418	0.393	NC	NC

Upper limit of quantification: 27.5 µg equiv/g for all measurements
Lower limit of quantification: 0.0183 µg equiv/g for all measurements

NC – Not calculated
*Tissues may be affected by high levels of radioactivity in the associated contents

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Table 5.1.1- 45: Terminal half-lives and AUC values of total radioactivity in the tissues of male rats after a single oral dose of 5 mg [phenyl-UL-^{14}C]BCS-CN88460/kg bw

Tissue	R^2	Number of time points used	$t_{1/2}$ (hours)	T_{max}	C_{max}	AUC (µg equiv.h/g)	
						0-_{last}	0-_{all}
Blood	NC	5	NC	4	0.464	7.99	8.39
Adrenal cortex	0.972	3	6.65	1	0.586	3.67	4.09
Adrenal medulla	0.955	3	15.4	1	0.666	5.12	5.40
Aortic wall	0.947	3	7.97	1	0.329	3.27	3.82
Bile ducts	1.00	3	10.4	1	5.25	102	104
Bone marrow	0.908	3	10.0	1	0.242	1.84	2.21
Bone surface	NC	NC	NC	1	0.024	0.08	0.120
Brain	NC	NC	NC	1	0.124	0.34	0.420
Brown fat	0.816	3	26.2	1	0.707	4.74	5.19
Bulbo-urethral gland	0.998	3	8.22	1	1.84	5.47	5.99
Caecum mucosa	0.994	3	10.6	4	7.39	36.5	37.0
Choroid plexus	NC	NC	NC	1	0.278	1.35	1.62
Epididymis	0.923	3	6.19	1	0.184	2.33	2.56
Exorbital lachrymal gland	0.997	3	6.63	1	0.567	3.29	3.63
Harderian gland	NC	NC	NC	1	0.438	1.77	2.34
Intra-orbital lachrymal gland	0.980	3	7.99	1	0.399	3.05	3.51
Large intestine mucosa	NC	NC	NC	24	0.803	27.1	27.9
Lens	NC	NC	NC	NC	NC	NC	NC
Liver	1.00	3	33.6	1	3.72	55.6	57.1
Lung	0.967	3	23.4	1	0.530	5.78	6.32
Mandibular lymph nodes	NC	NC	NC	1	0.314	1.43	2.01
Meninges	NC	NC	NC	1	0.135	0.790	1.49
Muscle	NC	NC	NC	1	0.197	0.820	1.22
Myocardium	0.883	4	7.26	1	0.533	3.57	4.06
Nasal mucosa	NC	5	NC	4	0.268	7.93	8.95
Oesophageal wall	NC	NC	NC	1	0.269	1.64	2.61
Pancreas	0.961	3	6.46	1	0.742	3.20	3.49
Periodontal membrane	0.903	3	7.29	1	0.246	1.74	1.98
Peri-renal fat	NC	NC	NC	1	0.134	0.60	0.840
Pineal body	NC	NC	NC	4	0.279	1.66	2.47
Pituitary gland	NC	NC	NC	1	0.239	1.05	1.46
Preputial gland	NC	NC	NC	24	1.51	75.1	95.0
Prostate	NC	NC	NC	4	0.339	2.52	2.79
Rectum mucosa	0.952	3	206	1	0.193	3.15	3.63
Renal cortex	0.899	5	15.6	1	1.13	12.8	13.7
Renal medulla	0.993	4	11.9	1	1.05	8.16	8.47
Salivary glands	0.828	3	7.45	1	0.420	2.21	2.50
Seminal vesicles	NC	NC	NC	1	0.247	2.39	4.08

NC – Tissue half-life not calculated as insufficient data points in the elimination phase available, or coefficient of determination (R^2) was <0.8

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Table 5.1.1- 45 continued

Tissue	R^2	Number of time points used	$t_{1/2}$ (hours)	T_{max}	C_{max}	AUC (µg equiv.h/g)	
						0-_{last}	0-_{all}
Skin (Non-pigmented)	NC	NC	NC	1	0.225	0.980	1.51
Small intestine mucosa	NC	4	NC	1	8.51	26.2	27.4
Spinal cord	NC	NC	NC	1	0.137	0.330	0.430
Spleen	NC	NC	NC	1	0.408	1.00	1.95
Stomach mucosa (fundus)	NC	NC	NC	4	0.706	5.55	7.06
Stomach mucosa (non-fundic)	0.992	3	5.32	1	14.9	34.0	34.5
Testis	NC	NC	NC	1	0.152	0.820	1.36
Thymus	NC	NC	NC	1	0.301	0.750	0.960
Thyroid	NC	NC	NC	1	0.348	1.54	2.16
Tongue	0.933	3	6.45	1	0.468	2.60	2.87
Tooth pulp	0.833	3	6.80	1	0.412	3.29	3.74
Trachea	NC	NC	NC	4	0.371	1.54	1.90
Urinary bladder wall	0.871	5	26.1	4	16.1	94.8	96.9
Urine	NC	5	NC	4	13.5	95.4	97.6
Uveal tract/retina	0.988	3	8.23	1	0.229	2.30	2.69
White fat	NC	NC	NC	1	0.117	0.390	0.590

NC – Tissue half-life not calculated as insufficient data points in the elimination phase available, or coefficient of determination (R^2) was <0.8

B. Distribution of radioactivity in female rats

Peak concentrations of radioactivity in the tissues of female rats typically occurred at the first sampling time of 1 hour, exceptions were the bile ducts where peak concentrations were measured 8 hours post-dose and the clitoris, where peak concentrations were observed 48 hours after dosing.

All investigated tissues were exposed to radioactive drug-related material at one or more of the sampling times. Radioactivity concentrations declined over the duration of the study so that at the final sampling time (168 hours) only 6% of the investigated tissues including clitoris (0.675 µg equiv/g), nasal mucosa (0.0382 µg equiv/g) and liver (0.0344 µg equiv/g) remained above the limits of quantification.

Concentrations of drug-related radioactivity in blood were 0.692, 0.318, 0.241, 0.0471 and 0.0461 µg equiv/g at 1, 4, 8, 24 and 120 hours after dosing, respectively. Radioactivity concentrations in blood were not quantifiable at 48, 72 or 168 hours post-dose.

Highest concentrations of radioactivity (>10 µg equiv/g to a maximum of 19.5 µg equiv/g at one or more sampling time) were observed in the adrenal cortex, bile ducts, brown fat, Harderian gland, liver, myocardium, nasal mucosa, ovary, pancreas, peri-renal fat, renal cortex and renal medulla. Lowest concentrations, at each sampling time, were associated with the brain, spinal cord, bone surface and lens of the eye.

The elimination half-lives of radioactivity from tissues, where reliable measurements could be ascertained, were generally less than 10 hours. The only exceptions, where half-lives were >10 hours, were observed in liver (77.8 hours) and bone marrow (10.2 hours).

$AUC_{0\text{-}last}$ values were typically below 50 µg equiv.h/g for the majority of tissues. Tissues exhibiting higher exposures ($AUC_{0\text{-}last}$) to drug-related radioactivity were the bile ducts (166 µg equiv.h/g), liver (67.1 µg equiv.h/g) and clitoris (62.4 µg equiv.h/g). $AUC_{0\text{-}last}$ values of <2.00 µg equiv.h/g, i.e. ca 6-fold lower than plasma, were determined in the mammary tissue, muscle, thymus, brain, spinal cord, bone surface and lens of the eye.

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Table 5.1.1- 46: Distribution of radioactivity in organs and tissues of female rats after a single oral dose of 5 mg [phenyl-UL-^{14}C]BCS-CN88460/kg bw

	µg equivalents of BCS-CN88460/g of tissue							
Animal Number and Sex	**241F**	**240F**	**235F**	**236F**	**237F**	**238F**	**239F**	**242F**
Tissue Sampling time (hours)	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Blood	0.692	0.318	0.241	0.0471	BLQ	BLQ	0.0461	BLQ
Adrenal cortex	1.42	0.459	0.325	0.0261	BLQ	BLQ	BLQ	BLQ
Adrenal medulla	0.976	0.415	0.237	0.0236	BLQ	BLQ	BLQ	BLQ
Aortic wall	0.639	0.306	0.249	0.0442	BLQ	BLQ	BLQ	BLQ
Bile ducts	0.972	6.23	19.5	0.892	0.0244	ND	0.0372	ND
Bone marrow	0.803	0.178	0.171	0.0494	ND	BLQ	ND	ND
Bone surface	0.0338	0.0252	BLQ	BLQ	ND	ND	ND	ND
Brain	0.350	0.0599	0.0471	BLQ	BLQ	BLQ	BLQ	BLQ
Brown fat	1.54	0.499	0.285	0.0503	BLQ	BLQ	BLQ	BLQ
Caecum mucosa	0.857	0.0906	0.335	1.80*	BLQ	BLQ	BLQ	BLQ
Choroid plexus	0.494	0.195	0.176	0.0511	BLQ	ND	0.0366	ND
Clitoris	0.293	0.439	0.277	0.457	0.943	BLQ	0.236	0.675
Exorbital lachrymal gland	0.758	0.243	0.237	0.0338	BLQ	BLQ	BLQ	BLQ
Harderian gland	1.06	0.323	0.245	0.0339	BLQ	BLQ	ND	BLQ
Intra-orbital lachrymal gland	0.731	0.265	0.246	0.0407	BLQ	BLQ	BLQ	BLQ
Large intestine mucosa	0.576	0.206	0.143	2.14*	0.0184	BLQ	BLQ	BLQ
Lens	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Liver	6.15	3.60	2.54	0.666	0.123	0.0809	0.0487	0.0344
Lung	0.826	0.327	0.279	0.0599	BLQ	BLQ	BLQ	BLQ
Mammary tissue	0.549	0.226	0.0773	BLQ	BLQ	BLQ	BLQ	BLQ
Mandibular lymph nodes	0.474	0.175	0.114	0.0286	BLQ	BLQ	BLQ	BLQ
Meninges	0.450	0.268	0.218	0.0317	BLQ	ND	0.0197	BLQ
Muscle	0.500	0.112	0.0943	BLQ	BLQ	BLQ	BLQ	BLQ
Myocardium	1.05	0.348	0.228	0.047	BLQ	BLQ	BLQ	BLQ
Nasal mucosa	15.8	0.121	0.783	0.0352	0.0218	BLQ	0.0447	0.0382
Oesophageal wall	0.628	0.215	0.183	0.0238	BLQ	BLQ	BLQ	BLQ
Ovary	1.05	0.290	0.444	0.0892	BLQ	BLQ	BLQ	BLQ
Pancreas	1.09	0.314	0.264	0.0781	BLQ	BLQ	BLQ	BLQ
Periodontal membrane	0.272	0.136	0.149	0.0191	BLQ	ND	BLQ	BLQ
Peri-renal fat	1.05	0.0945	0.264	0.0516	BLQ	BLQ	BLQ	BLQ
Pineal body	0.443	0.207	0.195	0.0391	BLQ	BLQ	BLQ	BLQ
Pituitary	0.736	0.184	0.165	0.0275	BLQ	ND	ND	ND
Rectum mucosa	0.537	0.0594	0.110	0.700*	BLQ	BLQ	BLQ	BLQ
Renal cortex	1.50	0.754	0.555	0.158	BLQ	BLQ	BLQ	BLQ
Renal medulla	1.39	0.439	0.551	0.0879	BLQ	BLQ	BLQ	BLQ
Salivary glands	0.873	0.247	0.219	0.0392	BLQ	BLQ	BLQ	BLQ

Upper limit of quantification: 275 µg equiv/g for all measurements
Lower limit of quantification: 0.0183 µg equiv/g for all measurements
BLQ - Concentration below the lower limit of quantification
ND – Radioactivity not detected

*Tissues may be affected by high levels of radioactivity in the associated contents

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Table 5.1.1- 46 continued

	µg equivalents of BCS-CN88460/g of tissue							
Animal Number and Sex	**241F**	**240F**	**235F**	**236F**	**237F**	**238F**	**239F**	**242F**
Tissue Sampling time (hours)	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Skin (Non-pigmented)	0.433	0.182	0.132	0.0241	BLQ	BLQ	BLQ	BLQ
Small intestine mucosa	0.793*	0.710	0.980	0.120	BLQ	BLQ	BLQ	BLQ
Spinal cord	0.376	0.087	ND	BLQ	BLQ	ND	BLQ	ND
Spleen	0.577	0.194	0.0533	0.0344	BLQ	BLQ	BLQ	BLQ
Stomach mucosa (fundus)	1.32*	0.344	0.284	0.0935	BLQ	BLQ	BLQ	BLQ
Stomach mucosa (non-fundic)	0.566	0.545	0.510	0.124	BLQ	BLQ	BLQ	BLQ
Thymus	0.414	0.117	0.105	BLQ	BLQ	BLQ	BLQ	BLQ
Thyroid	0.670	0.178	0.170	0.0313	BLQ	BLQ	BLQ	BLQ
Tongue	0.780	0.270	0.228	0.0368	BLQ	BLQ	BLQ	BLQ
Tooth pulp	0.316	0.235	0.265	0.0517	BLQ	ND	ND	BLQ
Trachea	0.411	0.153	0.107	0.0220	BLQ	BLQ	BLQ	BLQ
Urinary bladder wall	2.66	12.3	2.31	2.32	BLQ	ND	ND	ND
Urine	4.28	7.99	4.48	1.79	0.0185	BLQ	BLQ	BLQ
Uterus	0.672	0.272	0.255	0.220	BLQ	BLQ	BLQ	BLQ
Uveal tract/retina	0.466	0.227	0.160	0.0295	BLQ	BLQ	BLQ	ND
White fat	0.293	0.191	0.188	0.0367	BLQ	BLQ	BLQ	BLQ

Upper limit of quantification: 27.5 µg equiv/g for all measurements
Lower limit of quantification: 0.0183 µg equiv/g for all measurements
BLQ - Concentration below the lower limit of quantification
ND – Radioactivity not detected
*Tissues may be affected by high levels of radioactivity in the associated contents

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Table 5.1.1- 47: Tissue : blood ratios of radioactivity in the tissues of female rats after a single oral dose of 5 mg [phenyl-UL-^{14}C]BCS-CN88460/kg bw

	µg equivalents of BCS-CN88460/g of tissue							
Animal Number and Sex	**241F**	**240F**	**235F**	**236F**	**237F**	**238F**	**239F**	**242F**
Tissue **Sampling time (hours)**	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Blood	1.00	1.00	1.00	1.00	NC	NC	1.00	NC
Adrenal cortex	2.05	1.44	1.35	0.555	NC	NC	0.138	NC
Adrenal medulla	1.41	1.30	0.983	0.501	NC	NC	0.212	NC
Aortic wall	0.924	0.960	1.03	0.937	NC	NC	0.191	NC
Bile ducts	1.41	19.6	81.0	18.9	NC	NC	0.808	NC
Bone marrow	1.16	0.561	0.709	1.05	NC	NC	NC	NC
Bone surface	0.0488	0.0792	0.0101	0.175	NC	NC	NC	NC
Brain	0.506	0.188	0.195	0.0489	NC	NC	0.0655	NC
Brown fat	2.22	1.57	1.60	1.07	NC	NC	0.308	NC
Caecum mucosa	1.24	0.285	1.39	38.3*	NC	NC	0.201	NC
Choroid plexus	0.714	0.613	0.732	1.08	NC	NC	0.795	NC
Clitoris	0.423	1.38	1.15	9.71	NC	NC	5.12	NC
Exorbital lachrymal gland	1.10	0.763	0.982	0.718	NC	NC	0.133	NC
Harderian gland	1.54	1.01	1.02	0.720	NC	NC	NC	NC
Intra-orbital lachrymal gland	1.06	0.832	1.02	0.863	NC	NC	0.167	NC
Large intestine mucosa	0.832	0.647	0.595	45.3*	NC	NC	0.160	NC
Lens	NC	0.0460	0.0226	NC	NC	NC	0.0191	NC
Liver	8.89	11.3	10.9	14.1	NC	NC	1.06	NC
Lung	1.19	1.19	1.16	1.27	NC	NC	0.377	NC
Mammary tissue	0.751	0.711	0.321	0.371	NC	NC	0.165	NC
Mandibular lymph nodes	0.685	0.550	0.471	0.606	NC	NC	0.248	NC
Meninges	0.651	0.842	0.903	0.673	NC	NC	0.427	NC
Muscle	0.723	0.353	0.391	0.370	NC	NC	0.0821	NC
Myocardium	1.51	1.09	0.948	0.997	NC	NC	0.396	NC
Nasal mucosa	22.9	0.379	3.25	0.746	NC	NC	0.971	NC
Oesophageal wall	0.908	0.675	0.759	0.505	NC	NC	0.211	NC
Ovary	1.52	0.910	1.84	1.89	NC	NC	0.174	NC
Pancreas	1.58	0.986	1.10	1.66	NC	NC	0.166	NC
Periodontal membrane	0.393	0.428	0.618	0.406	NC	NC	0.0504	NC
Peri-renal fat	1.52	0.297	1.09	1.09	NC	NC	0.0801	NC
Pineal body	0.641	0.651	0.807	0.830	NC	NC	0.125	NC
Pituitary	1.07	0.579	0.684	0.584	NC	NC	NC	NC
Rectum mucosa	0.769	0.187	0.455	14.8*	NC	NC	0.0538	NC
Renal cortex	2.16	2.37	2.30	3.35	NC	NC	0.350	NC
Renal medulla	2.02	1.38	2.28	1.87	NC	NC	0.242	NC
Salivary glands	1.26	0.774	0.909	0.831	NC	NC	0.0638	NC

Upper limit of quantification: 27.5 µg equiv/g for all measurements
Lower limit of quantification: 0.0183 µg equiv/g for all measurements
NC – Not calculated
*Tissues may be affected by high levels of radioactivity in the associated contents

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Table 5.1.1- 47 continued

	µg equivalents of BCS-CN88460/g of tissue							
Animal Number and Sex	**241F**	**240F**	**235F**	**236F**	**237F**	**238F**	**239F**	**242F**
Tissue **Sampling time (hours)**	**1**	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Skin (Non-pigmented)	0.626	0.572	0.547	0.511	NC	NC	0.233	NC
Small intestine mucosa	1.15*	2.23	4.06	2.55	NC	NC	0.185	NC
Spinal cord	0.544	0.273	NC	0.0389	NC	NC	0.105	NC
Spleen	0.834	0.610	0.221	0.731	NC	NC	0.147	NC
Stomach mucosa (fundus)	1.91*	1.08	1.18	1.99	NC	NC	0.164	NC
Stomach mucosa (non-fundic)	0.819	1.71	2.12	2.64	NC	NC	0.156	NC
Thymus	0.599	0.368	0.437	0.290	NC	NC	0.144	NC
Thyroid	0.969	0.558	0.705	0.664	NC	NC	0.322	NC
Tongue	1.13	0.848	0.944	0.780	NC	NC	0.225	NC
Tooth pulp	0.458	0.738	1.10	1.10	NC	NC	NC	NC
Trachea	0.595	0.480	0.442	0.466	NC	NC	0.320	NC
Urinary bladder wall	3.85	38.6	9.58	49.3	NC	NC	NC	NC
Urine	6.18	25.1	18.6	38.1	NC	NC	0.251	NC
Uterus	0.972	0.855	1.47	4.66	NC	NC	0.0974	NC
Uveal tract/retina	0.674	0.714	0.665	0.627	NC	NC	0.156	NC
White fat	0.422	0.599	0.780	0.780	NC	NC	0.0971	NC

Upper limit of quantification: 27.3 µg equiv/g for all measurements
Lower limit of quantification: 0.0183 µg equiv/g for all measurements
NC – Not calculated
*Tissues may be affected by high levels of radioactivity in the associated contents

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Table 5.1.1- 48: Terminal half-lives and AUC values of total radioactivity in the tissues of female rats after a single oral dose of 5 mg [phenyl-UL-^{14}C]BCS-CN88460/kg bw

Tissue	R^2	Number of time points used	$t_{1/2}$ (hours)	T_{max}	C_{max}	AUC (µg equiv.h/g)	
						0-_{last}	0-_{all}
Blood	0.997	3	7.12	1	0.692	6.47	7.58
Adrenal cortex	0.994	3	4.70	1	1.42	6.71	7.02
Adrenal medulla	1.00	3	4.83	1	0.976	5.21	5.49
Aortic wall	0.991	3	6.93	1	0.639	4.68	5.21
Bile ducts	NC	3	NC	1	19.5	166	167
Bone marrow	0.974	3	10.2	1	0.803	3.91	4.50
Bone surface	NC	NC	NC	1	0.034	0.100	0.160
Brain	NC	NC	NC	1	0.350	0.880	1.26
Brown fat	0.992	3	5.86	1	1.54	7.93	8.53
Caecum mucosa	NC	NC	NC	24	1.80	19.8	41.4
Choroid plexus	0.985	3	9.91	1	0.494	5.06	5.94
Clitoris	NC	NC	NC	48	0.943	62.4	62.4
Exorbital lachrymal gland	0.968	3	6.39	1	0.758	4.37	4.77
Harderian gland	0.994	3	5.98	1	1.06	5.23	5.63
Intra-orbital lachrymal gland	0.976	3	7.00	1	0.731	4.59	5.08
Large intestine mucosa	NC	NC	NC	24	2.14	31.1	31.4
Lens	NC	NC	NC	NC	NC	NC	NC
Liver	0.988	3	77.8	1	6.15	67.1	67.1
Lung	0.999	3	7.44	1	0.826	5.71	6.43
Mammary tissue	NC	NC	NC	1	0.519	1.87	2.49
Mandibular lymph nodes	0.999	3	7.75	1	0.474	2.69	3.04
Meninges	0.990	3	6.26	1	0.450	4.65	5.12
Muscle	NC	NC	NC	1	0.500	1.44	2.19
Myocardium	1.00	3	6.95	1	1.05	5.40	5.96
Nasal mucosa	0.757	3	8.25	1	15.8	26.8	26.8
Oesophageal wall	0.985	3	6.03	1	0.628	3.51	3.80
Ovary	0.811	3	9.79	1	1.05	7.28	8.35
Pancreas	0.994	3	9.70	1	1.09	6.01	6.95
Periodontal membrane	0.948	3	6.49	1	0.272	2.31	2.54
Peri-renal fat	NC	NC	NC	1	1.05	4.46	5.08
Pineal body	0.975	3	7.86	1	0.443	3.51	3.98
Pituitary	0.981	3	6.94	1	0.736	3.49	3.82
Rectum mucosa	NC	NC	NC	24	0.700	7.97	16.4
Renal cortex	1.00	3	8.86	1	1.50	11.7	13.6
Renal medulla	0.910	3	7.68	1	1.39	9.18	10.2
Salivary glands	0.983	3	7.19	1	0.873	4.53	5.00

NC – Tissue half-life not calculated as insufficient data points in the elimination phase available, or coefficient of determination (R^2) was < 0.8

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Table 5.1.1- 48 continued

Tissue	R^2	Number of time points used	$t_{1/2}$ (hours)	T_{max}	C_{max}	AUC (µg equiv.h/g)	
						0-_{last}	0-_{all}
Skin (Non-pigmented)	0.998	3	6.76	1	0.433	2.72	3.01
Small intestine mucosa	NC	NC	NC	8	0.980	12.6	14.0
Spinal cord	NC	NC	NC	1	0.376	0.780	0.95
Spleen	NC	NC	NC	1	0.577	2.47	2.88
Stomach mucosa (fundus)	0.997	3	10.4	1	1.32	6.83	7.95
Stomach mucosa (non-fundic)	0.978	3	8.87	1	0.566	8.43	9.91
Thymus	NC	NC	NC	1	0.414	1.36	2.20
Thyroid	0.972	3	7.51	1	0.670	3.46	3.83
Tongue	0.987	3	6.68	1	0.780	4.50	4.94
Tooth pulp	0.936	3	8.33	1	0.316	4.07	4.69
Trachea	1.00	3	7.11	1	0.411	2.36	2.63
Urinary bladder wall	NC	NC	NC	4	12.3	84.7	113
Urine	0.938	3	4.90	4	7.99	101	101
Uterus	NC	3	NC	1	0.672	7.42	10.1
Uveal tract/retina	0.999	3	6.73	1	0.466	3.23	3.59
White fat	0.967	3	7.87	1	0.293	3.10	3.54

NC – Tissue half-life not calculated as insufficient data points in the elimination phase available, or coefficient of determination (R^2) was <0.8

C. Recovery of radioactivity

The amount of radioactivity recovered in the excreta prior to sacrifice was determined. Mass balance could not be established for any male animals, although full recovery (>90%) was obtained for the animals sacrificed at 72, 120 and 168 hours, as the carcasses were retained for QWBA assessment. Mass balance could not be established for any female animals as the carcasses were retained for QWBA assessment.

However, results indicated that less than 5% of the administered dose was eliminated in the first 8 hours post-dose for both male and female rats. The elimination increased to approximately 70% for male rats and to 40% for female rats by 24 hours post-dose. By 48 hours, and thereafter, ca 90% of the administered radioactivity had been recovered in the excreta of male rats and >80% in the excreta of female rats. The major route of elimination was via the faeces with 80% or more (male rats) and 68-80% (female rats) eliminated in the faeces by 48 hours. Results from the WBA analysis suggest that biliary secretion was a contributing factor in faecal elimination with radioactivity present in bile duct contents at 1, 4, 8, 24, 48, 72 and 120 hours post-dose.

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Table 5.1.1- 49: Cumulative excretion of radioactivity in urine, faeces, cage wash and cage debris of male rats after a single oral administration of 5 mg [phenyl-UL-^{14}C]BCS-CN88460/kg bw

Sample	Collection interval (hours)	Recovery (% administered dose)							
		101M	102M	103M	104M	105M	106M	107M	108M
Urine	0-1	NS	-	-	-	-	-	-	-
	1-4	-	0.216	0.186	0.61	0.827	1.10	0.0668	0.378
	4-8	-	-	2.97	2.26	2.42	1.73	1.49	2.05
	8-24	-	-	-	4.93	4.55	3.71	4.02	4.82
	24-48	-	-	-	-	5.24	4.35	4.91	5.66
	48-72	-	-	-	-	-	4.57	5.25	5.94
	72-96	-	-	-	-	-	-	5.38	6.02
	96-120	-	-	-	-	-	-	5.43	6.09
	120-144	-	-	-	-	-	-	-	6.13
	144-168	-	-	-	-	-	-	-	6.17
	Subtotal	NA	0.216	2.97	4.93	5.24	4.57	5.43	6.17
Faeces	0-24	-	-	-	74.3	66.0	76.2	60.5	65.2
	24-48	-	-	-	-	79.3	85.6	84.0	83.3
	48-72	-	-	-	-	-	87.4	88.5	85.2
	72-96	-	-	-	-	-	-	89.6	85.5
	96-120	-	-	-	-	-	-	89.8	85.5
	120-144	-	-	-	-	-	-	-	85.6
	144-168	-	-	-	-	-	-	-	85.6
	Subtotal	-	-	-	74.3	79.3	87.4	89.8	85.6
Cage wash	0-24	-	-	-	-	0.758	0.573	0.353	0.709
	24-48	-	-	-	-	-	0.864	2.12	1.01
	48-72	-	-	-	-	-	-	2.19	1.15
	72-96	-	-	-	-	-	-	2.22	1.23
	96-120	-	-	-	-	-	-	-	1.26
	120-144	-	-	-	-	-	-	-	1.29
	Subtotal	-	-	-	-	0.758	0.864	2.22	1.29
Cage debris	168	-	-	-	0.0150	0.0282	0.0361	0.0511	0.0562
Final cage wash	168	-	-	-	0.637	0.969	0.285	0.0819	0.0628
	Total	NA	0.216	2.97	79.9	86.3	93.1	97.6	93.1

NS: No Sample
NA: Not Applicable

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Table 5.1.1- 50: Cumulative excretion of radioactivity in urine, faeces, cage wash and cage debris of female rats after a single oral administration of 5 mg [phenyl-UL-^{14}C]BCS-CN88460/kg bw

Sample	Collection interval (hours)	Recovery (% administered dose)							
		109F	110F	111F	112F	113F	114F	115F	116F
Urine	0-1	0.109	-	-	-	-	-	-	-
	1-4	-	1.54	0.307	1.09	1.97	0.973	0.0798	0.207
	4-8	-	-	3.15	2.12	3.38	1.78	1.39	0.861
	8-24	-	-	-	3.50	5.58	3.38	5.67	3.83
	24-48	-	-	-	-	6.18	4.16	6.38	4.87
	48-72	-	-	-	-	-	4.45	6.60	5.06
	72-96	-	-	-	-	-	-	6.65	5.19
	96-120	-	-	-	-	-	-	6.67	5.24
	120-144	-	-	-	-	-	-	-	5.28
	144-168	-	-	-	-	-	-	-	5.33
	Subtotal	0.109	1.54	3.15	3.50	6.18	4.45	6.67	5.33
Faeces	0-24	-	-	-	36.4	63.6	55.5	43.1	37.4
	24-48	-	-	-	-	83.8	83.1	68.0	74.0
	48-72	-	-	-	-	-	87.4	70.6	76.2
	72-96	-	-	-	-	-	-	71.1	76.7
	96-120	-	-	-	-	-	-	71.2	77.1
	120-144	-	-	-	-	-	-	-	77.4
	144-168	-	-	-	-	-	-	-	77.7
	Subtotal	-	-	-	36.4	83.8	87.4	71.2	77.7
Cage wash	0-24	-	-	-	-	0.639	0.535	0.365	0.353
	24-48	-	-	-	-	-	0.860	0.530	0.596
	48-72	-	-	-	-	-	-	0.609	0.744
	72-96	-	-	-	-	-	-	0.676	0.771
	96-120	-	-	-	-	-	-	-	0.835
	120-144	-	-	-	-	-	-	-	0.900
	Subtotal	-	-	-	-	0.639	0.860	0.676	0.900
Cage debris	168	-	-	-	0.00460	0.0116	0.0501	0.0320	0.0369
Final cage wash	168	-	-	-	0.942	0.471	0.326	1.05	1.09
	Total	0.109	1.54	3.15	40.8	91.1	93.1	79.6	85.0

D. Plasma kinetics

Plasma was collected from the male and female animals scheduled for sacrifice at 168 hours at 0.25, 1, 2, 7, 24, 48, 72, 96, 120, 144 and 168 hours post-dose. The concentrations of [phenyl-UL-14C]BCS-CN88460 in terms of µg equivalents/g and the pharmacokinetic parameters were determined. Peak concentrations (Cmax) in plasma of 1.038 and 0.800 µg equiv/g were measured at 1 and 2 hours in male and female animals, respectively. In male animals radioactivity was present in plasma at all sampling times except 144 hours where the concentration was below the limit of detection and in female animals radioactivity was not detected beyond 96 hours. The elimination half-life was 18.4 hours in male plasma and 12.8 hours in female plasma with corresponding AUC_{last} determined as 12.4 and 12.2 µg equiv.h/g, respectively.

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Table 5.1.1- 51: Concentrations of radioactivity in the plasma of male and female rats after a single oral administration of 5 mg [phenyl-UL-^{14}C]BCS-CN88460/kg bw

	µg equivalents of BCS-CN88460/g	
Animal Number and Sex	**108M**	**116F**
Timepoint (hour)		
0.25	0.261	0.156
1	1.038	0.724
2	0.855	0.800
7	0.583	0.609
24	0.100	0.146
48	0.031	0.023
72	0.023	0.009
96	0.008	0.005
120	0.006	ND
144	ND	ND
168	0.005	ND

ND – Not detected

III. CONCLUSIONS

Radioactivity was widely distributed following oral administration.

Elimination of drug-related radioactivity was rapid. The majority of tissues did not contain quantifiable radioactivity at the final sampling time of 168 hours post-dose.

Highest radioactivity concentrations, excluding tissues of the gastrointestinal tract, were determined in the liver, renal cortex and renal medulla.

Elimination half-lives of radioactivity were typically <20 hours. Elimination half-lives of >20 hours were observed in the brown fat, liver and lung of male animals and in the liver of female animals.

AUC values were generally <10 µg equiv.h/g, but were higher (up to 166 µg equiv.h/g) for some tissues (bile ducts, preputial gland (males only), clitoris (females only), liver and renal cortex).

The detection of low levels of radioactivity in the central nervous system at early sampling times (4, 8 and 24 hours) suggested that drug-related material crossed the blood-brain barrier, but was subsequently eliminated.

Elimination of radioactivity was generally via faecal voiding. The presence of high levels of radioactivity in the bile ducts indicated that biliary secretion was a contributing factor in faecal elimination.

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Report:	KCA 5.1.1/05; [redacted], R., [redacted], N.; 2017; M-604147-02-1
Title:	Amendment no. 1: [Pyrazolyl-4-14C]BCS-CY26497: Pilot metabolism experiments in male rats
Report No.:	EnSa-16-1013
Document No.:	M-604147-02-1
Guideline(s):	Regulation (EC) No 1107/2009 amended by Commission Regulation (EU) No 283/2013 OECD Guideline for the Testing of Chemicals, 417 US EPA OCSPP Health Effects Test Guidelines, 870.7485 Japan/FAMIC-ACIS Notification 12-Nousan 8147
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

The absorption, excretion, and metabolism of [pyrazolyl-4-^{14}C]BCS-CY26497 (BCS-CN88460-carboxylic acid), one of the major metabolites of BCS-CN88460, were investigated in male Wistar rats. The rats received a single target dose of about 5 mg of the test compound per kilogram body weight by oral gavage.

The elimination of radioactivity from blood was analysed quantitatively by collecting micro samples from one single rat at individual time points. In addition, the amount of radioactivity in excreta (urine and/or faeces) was determined for selected time periods in all rats. One rat each was sacrificed at 1, 4, 8, 24, and 48 h after administration. Urine, faeces, plasma, liver and kidney samples were collected.

[Pyrazolyl-4-^{14}C]BCS-CY26497 was absorbed very fast from the gastrointestinal tract of male Wistar rats after a single oral dose of 5 mg/kg bw. The plasma maximum was reached immediately after dosing and measured for 4.7157 mg/kg at 0.25 h. The majority of the radioactivity administered was excreted *via* the faeces and was 103.68% of dose. Excretion *via* urine was 1.52% of dose, only. The excretion was nearly complete within 24 h after administration and amounted to 104.14% of dose.

Urine and the conventional faeces extract from one selected animal were investigated by HPLC.

BCS-CY26497 and its metabolites were identified by comparison of the metabolic profiles form urine and faeces extract of the current study with the profiles of urine and bile analysed in the rat ADME study with the pyrazole label ([redacted], R.; [redacted], N.; 2017; M-602883-02-1), in which the components were identified by spectroscopic methods.

The following metabolites based on BCS-CY26497 (BCS-CN88460-carboxylic acid) have been identified:

- BCS-CN88460-desmethyl-carboxylic acid
- BCS-CN88460-desmethyl-lactic acid
- BCS-CN88460-lactic acid
- BCS-CN88460-desmethyl-hydroxymethyl-carboxylic acid
- BCS-CN88460-pyrazole-carboxylic acid-Ala
- BCS-CN88460-cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)
- BCS-CN88460-N-methyl-pyrazole-carboxylic acid
- BCS-CN88460-cyclopropyl-oxy-pyrazole-carboxamide
- BCS-CN88460-cyclopropyl-pyrazole-carboxamide
- BCS-CN88460-cyclopropyl-pyrazole-carboxamide-OH-GlucA
- BCS-CN88460-pyrazole-amide
- BCS-CN88460-pyrazole-carboxylic acid

In all samples investigated (urine and extract of faeces), parent compound was the most prominent component (58.6% of dose). BCS-CN88460-desmethyl-carboxylic acid was identified and characterised as major metabolite and amounted to 31.5% of dose. BCS-CN88460-desmethyl-lactic

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acid (2.9%) and BCS-CN88460-lactic acid (2.4%) were predominant. Another prominent metabolite (peak ID: F32) occurred to 5.8% in the extract of faeces but was characterised by its chromatographic behaviour, only. All other identified metabolites were detected in very low amounts (≤ 0.6% of dose).

The principal metabolic reactions of [pyrazolyl-4-^{14}C]BCS-CY26497 in the rat are listed below:

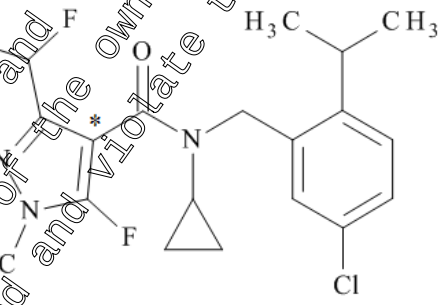
- demethylation of the pyrazole moiety
- hydroxylation in position 2 of the propionic acid group and after defluorination of the difluoromethyl moiety was leading to compounds with a lactic acid group and a hydroxymethyl group. Hydroxylation of BCS-CN88460-cyclopropyl-pyrazole-carboxamide was also detected, but was not exactly located by structure elucidation.
- cleavage of the phenyl moiety was leading to cyclopropyl-pyrazole-carboxamide compounds
- cleavage of the phenyl moiety in combination with cleavage of the cyclopropyl ring was leading to a pyrazole-amide compound followed by oxidation to a carboxylic acid compound
- conjugation of BCS-CN88460-cyclopropyl-pyrazole-carboxamide with glucuronic acid via nitrogen or after hydroxylation was leading to glucuronic acid conjugates
- conjugation of BCS-CN88460-pyrazole-carboxylic acid compound with alanine
- oxidation in the pyrazole ring was leading to BCS-CN88460-cyclopropyl-oxy-pyrazole-carboxamide

From these pilot metabolism investigations a metabolite pathway can be depicted.

I. Material and Methods

A. Material

1. Test material:

Test substance	
Chemical structure	 *denotes the position of the ^{14}C-radiolabel
Radiolabelled test material	[Pyrazolyl-4-^{14}C]BCS-CY26497 (BCS-CN88460-carboxylic acid)
Specific radioactivity	3.92 MBq/mg = 2.35 x 10^8 dpm/mg
Chemical purity	>99% (HPLC)
Radiochemical purity	>98% (HPLC); >98% (TLC)
Vehicle	0.5% aqueous Tragacanth solution
Preparation of dosing solution	radiolabelled test compound suspended in aqueous Tragacanth solution

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2. Test animals:

Species:	Rat (*Rattus norvegicus domesticus*)
Strain:	Wistar Hsd/Cpb: WU
Breeding facility:	[redacted]
Sex and numbers involved:	Males: 5 animals
Age:	about 6 - 7 weeks at the time of delivery
Body weight:	197 - 208 g at the time of administration 195 - 206 g at the time of sacrifice
Acclimatization:	During the acclimation periods, the animals were kept in Makrolon® cages on wood shavings for about 7 days prior to the administration.
Identification:	The animals were identified by cage cards on which the study number, test compound name and individual animal number were displayed. They were additionally labelled with water-insoluble spots on the tail.
Housing:	During the testing periods, the rats were kept individually in Makrolon® metabolism cages. With these cages, an almost quantitative and separate collection of urine and faeces was possible. While in cages, the animals had unrestricted access to food and water. Temperature 20.2 - 23.6 °C, relative humidity 29.6 - 68.8%, 12 / 12 hours light / dark cycle.
Feed and water:	The rats were fed ad libitum with ssniff® rat/mice maintenance feed (R/M-H Ered I, 10 mm pellets, article no. V1574-000, supplied by [redacted]). They received approximately 16 g per animal and day. Tap water from the local mains supply was given ad libitum (water specification in accordance to the local drinking water regulations).

B. Study design

1. Dosing

The suspension was administered to the rats by oral gavage using a syringe attached to an animal-feeding knob cannula. Each rat received 2 mL of the respective administration suspension of [pyrazolyl-4-^{14}C]BCS-CY26497. The volume was based on a nominal average animal weight of 204 g. The concentration of each administration suspension was calculated to reach a target dose of 5 mg per kg body weight of the rats. Due to different animal weights at administration, the mean actual dose in the tests amounted to 4.92 mg/kg body weight. The stability of the test compound in the suspensions was assured by radio-HPLC analysis after administration.

2. Sacrifice

The rats were sacrificed in deep pentobarbital-sodium anaesthesia by transection of the cervical vessels and exsanguination.

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3. Collection of excreta
Urine was collected separately for each animal in a cryogenic trap cooled with dry ice. Samples were collected separately for animal 383 (0 - 1 h), animal 384 (0 - 4 h), animal 385 (0 - 8 h), and animal 386 (0 - 24 h) at the time of sacrifice and for animal 387 (0 - 24 h and 24 - 48 h). The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinsing solutions were drained into the same vial as the corresponding urine fraction. The samples were weighed and kept frozen until analytical investigations. For determination of the total radioactivity by LSC all individual urine samples thawed, the respective aliquots removed from each sample, and the samples were kept frozen until further analytical investigations.
Faeces samples were collected separately for animal 385 (0 - 8 h), animal 386 (0 - 24 h), and animal 387 (0 - 24 h and 24 - 48 h). The samples were weighed and kept frozen. For combustion the samples were thawed, mixed with approximately the same volume of water, and homogenized. Five aliquots were analysed by combustion, the remainder individual samples were kept frozen until extraction and analytical investigations.

4. Preparation of blood/plasma samples
Micro plasma samples were taken at 0.25, 0.5, 1, 4, 8, 24, and 48 h from animal 387 only. Plasma and blood cells were separated by centrifugation. The content of radioactivity of the plasma samples was determined by LSC.

5. Plasma and organs at sacrifice
At the time of sacrifice the oozing out individual blood sample from each rat were collected from the cut wounds in heparinized test tubes and separated into plasma and blood cells by centrifugation. After weighing the samples were kept frozen. For radioactivity measurement by LSC plasma samples were thawed and aliquots were analysed. The residual samples were kept frozen until further analytical investigations. The individual samples of the blood cells were thawed and aliquots were taken for combustion. The residual samples were kept frozen until further analytical investigations.

Liver and kidney of all individual rats were weighed immediately after dissection. No separate determination of radioactivity in these organs was performed in order to have enough sample material for extraction and metabolic profiling. All samples were kept frozen until extraction and analytical investigations if considered appropriate.

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

C. Analytical procedures
Samples were analysed by radiochromatographic (HPLC) and spectroscopic (LC-MS/MS, ^{1}H-NMR) methods.

1. Preparation of samples, extraction and analysis
The collected plasma samples and the collected liver and kidney samples from each animal have not been analysed in the course of the study.
Urine samples were used for metabolic profiling without further sample preparation. The faeces samples of the sampling intervals 0-24 h were extracted with acetonitrile/water (8/2; v/v) mixtures using a homogeniser and were used for the quantitative analysis of parent compound and metabolites by HPLC.

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2. Identification / characterisation and quantification of residues

Parent compound and its metabolites in samples urine and in extracts of faeces were quantified by HPLC based on reversed phase chromatography using an acidic water/acetonitrile gradient. The peaks were numbered according to the peak IDs used in the rat ADME study with [pyrazole-4-14C]BCS-CN88460 ([redacted], R.; [redacted], N.; 2017; M-602452-02-1).

The identification of parent compound and metabolites was performed based on the comparison of the metabolic profiles of the current study with the profiles of the rat ADME study with the pyrazole label, in which the components were identified by spectroscopic methods. Unknown metabolites were characterised by their retention times.

II. RESULTS AND DISCUSSION

The absorption, excretion and metabolism of BCS-CY26497 (BCS-CN88460-carboxylic acid), one of the major metabolites of the fungicide BCS-CN88460, was investigated in male Wistar rats. BCS-CY26497 was labelled with ^{14}C in the pyrazolyl-4 moiety of the molecule. The rats received a single mean dose of 4.92 mg of the test compound per kilogram body weight by oral gavage.

A. Absorption of radioactivity

Samples of plasma and blood cells from each animal at the time of sacrifice were analysed after separation of whole blood by centrifugation. The total radioactivity in plasma samples and blood cell samples of all animals was determined based on combustion of aliquots and LSC measurements as shown below.

Table 5.1.1- 52: Total radioactivity in plasma and blood cells at sacrifice of male rats after a single oral administration of 5 mg [pyrazole-4-^{14}C]BCS-CY26497 /kg bw

Plasma at sacrifice				
Animal No.	Time after dosing [h]	Sample weight [g]	Radioactivity in aliquot [dpm/g]	TRR [mg eq/kg]
383	1	1.9853	603470	2.579
384	4	1.5038	379800	1.623
385	8	1.9592	46270	0.198
386	24	1.9594	21720	0.093
387	48	1.8431	21720	0.093
Blood cells (erythrocytes) at sacrifice				
Animal No.	Time after dosing [h]	Sample weight [g]	Radioactivity in aliquot [dpm/g]	TRR [mg eq/kg]
383	1	2.0438	65486	0.278
384	4	2.0802	25344	0.108
385	8	2.2334	18383	0.078
386	24	2.8748	4645	0.020
387	48	2.1343	6388	0.027

B. Time course of radioactivity concentration in the plasma

The maximum plasma level (C_{max}) of [pyrazolyl-4-^{14}C]BCS-CY26497 was measured at 0.25 h after administration. Dose normalised plasma levels decreased from 0.9246 at 0.25 h to 0.0092 at 48 h after dosing.

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Table 5.1.1- 53: Equivalent and dose normalised concentrations of plasma micro samples from one selected male rat (sacrificed 48 h after dosing) after a single oral administration of 5 mg [pyrazole-4-^{14}C]BCS-CY26497 /kg bw

Sampling time [h p admin.]	Equiv. concentration C [µg eq/g]	Dose normalised concentration C_{norm} *)
0.25	4.7157	0.9246
0.5	4.0252	0.7893
1	2.4024	0.4711
4	0.8195	0.1607
8	0.7936	0.1556
24	0.0355	0.0070
48	0.0469	0.0092

*) Animal 387 was dosed with 5.1 mg/kg body weight

C. Excretion of radioactivity

The main portion of radioactivity (102.74% of dose) was excreted via faeces within 24 h after administration and negligible amounts (0.94% of dose) were found in the time period 24 h to 48 h.
The total radioactivity excreted within the 48 h amounted to 1.52% of dose for urine and 103.86% of dose for faeces of the selected animal 387.

Table 5.1.1- 54: Total radioactivity in urine and faeces from one selected male rat (sacrificed 48 h after dosing) after a single oral administration of 5 mg [pyrazole-4-^{14}C]BCS-CY26497 /kg bw

Urine					
Animal no.	Time period [h]	Sample weight [g]	Radioactivity in aliquot [dpm/g]	Radioactivity total [dpm]	Radioactivity [% of dose administered]
387	0 - 24	21.27	155680	3311314	1.40
	24 - 48	31.25	9010	281563	0.12
Faeces					
Animal no.	Time period [h]	Sample weight [g]	Radioactivity in aliquot [dpm/g]	Radioactivity total [dpm]	Radioactivity [% of dose administered]
387	0 - 24	65.96	3682603	242904494	102.74
	24 - 48	62.29	35648	2220514	0.94

D. Quantitation of parent compound and metabolites

Parent compound and metabolites were quantified in the metabolite profiles from urine samples and extract of faeces of animal 387.
Urine samples were analysed without any sample preparation by HPLC. Faeces samples were conventionally extracted with ACN/water (8/2; v/v) mixtures. The extraction rates amounted to 98.8% for the sample collected during the period of 0 to 24 h after dosing. An aliquot of the conventional extract was analysed by HPLC.
In the urine samples up to 16 metabolites were detected beside parent compound. No component exceeded a value of 0.5% of dose.

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Parent compound was the largest fraction that was detected in the faeces extract of animal 387 and amounted to 58.6% of dose. BCS-CN88460-desmethyl-carboxylic acid was identified and characterised as major metabolite (31.4% of dose). BCS-CN88460-desmethyl-lactic acid (2.9% of dose) and BCS-CN88460-lactic acid (2.4% of dose) were predominant. The prominent metabolite F32 occurred to 5.8% of dose in the extract of faeces, but could not be identified by comparison of the metabolite profiles and was characterised by its chromatographic behaviour. Besides that, many other identified metabolites were detected and amounted to ≤ 0.51% of dose. Unknown metabolites were characterised based on their chromatographic behaviour.

Table 5.1.1- 55: Quantitative evaluation of parent compound and its metabolites in urine and faeces after a single oral dose of 5 mg [pyrazole-4-^{14}C] BCS-CY26497/kg bw (Animal 387)

Peak ID	Report name BCS-CN88460-	Animal 387 (male, 5 mg/kg bw)		
		Urine (0 - 24 h)	Faeces (0 - 24 h)	Total
		% of dose administered		
U1	pyrazole-amide	0.03	---	0.03
U3	cyclopropyl-pyrazole-carboxamide-OH-GlucA	0.04	---	0.04
U4	pyrazole-carboxylic acid	0.02	---	0.02
U7	cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)	0.24	---	0.24
U10	pyrazole-carboxylic acid-Ala	0.49	---	0.49
U11	N-methyl-pyrazole-carboxylic acid	0.14	---	0.14
U15	cyclopropyl-pyrazole-carboxamide	0.04	---	0.04
U18	cyclopropyl-oxy-pyrazole-carboxamide	0.06	---	0.06
F13	desmethyl-lactic acid	---	2.86	2.86
F14	desmethyl-hydroxymethyl-carboxylic acid	---	0.51	0.51
F16	lactic acid	---	2.39	2.39
U26, F20	desmethyl-carboxylic acid	0.07	31.40	31.47
U27, F23	BCS-CY26497-(BCS-CN88460-carboxylic acid)	0.04	58.57	58.61
Total identified		**1.18**	**95.72**	**96.89**
U5	unknown	0.04	---	0.04
U12	unknown	0.06	---	0.06
U13	unknown	0.02	---	0.02
U14	unknown	0.01	---	0.01
U17	unknown	0.02	---	0.02
U19	unknown	0.06	---	0.06
U25	unknown	0.02	---	0.02
F32	unknown	---	5.76	5.76
Total characterised		**0.22**	**5.76**	**5.99**
Solids of faeces				1.26
Urine not analysed (24 - 48 h)				0.12
Faeces not analysed (24 - 48 h)				0.94
Total				**105.20**

--- = not detected

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III. CONCLUSIONS

BCS-CY26497 (BCS-CN88460-carboxylic acid), one of the major metabolites of the insecticide BCS-CN88460, labelled with ^{14}C in the pyrazolyl-4 moiety of the molecule, was absorbed very fast from the gastrointestinal tract of male Wistar rats after single oral administration. The highest plasma level was measured at 0.25 hours. Excretion of radioactivity was all most via faeces and only a minor part with urine. Excretion was nearly completed within 24 hours.
BCS-CY26497 was the main compound in the extract of faeces. The major amount of follow-up metabolites from BCS-CY26497 was excreted predominantly via the faecal route, but the major number of metabolites was detected in the urine, representing very low amounts.
Demethylation of the pyrazole moiety and hydroxylation in the propionic acid group were the most prominent metabolic reaction. Cleavage of the phenyl moiety and the cyclopropyl ring were observed as prominent reactions. Further conjugation with glucuronic acid after hydroxylation or via nitrogen and conjugation with alanine was found.
Besides that, many minor metabolites had been formed showing that the chemical structure of the test compound is obviously changed at several positions of the molecule by metabolic reactions.
The following follow-up metabolites were identified:

- BCS-CN88460-desmethyl-carboxylic acid
- BCS-CN88460-desmethyl-lactic acid
- BCS-CN88460-lactic acid
- BCS-CN88460-desmethyl-hydroxymethyl-carboxylic acid
- BCS-CN88460-pyrazole-carboxylic acid-Ala
- BCS-CN88460-cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2)
- BCS-CN88460-N-methyl-pyrazole-carboxylic acid
- BCS-CN88460-cyclopropyl-oxy-pyrazole-carboxamide
- BCS-CN88460-cyclopropyl-pyrazole-carboxamide
- BCS-CN88460-cyclopropyl-pyrazole-carboxamide-OH-GlucA
- BCS-CN88460-pyrazole-amide
- BCS-CN88460-pyrazole-carboxylic acid

All results of the current study were in good conformation with the observations made in the rat ADME study with BCS-CN88460 using the pyrazole label ([redacted], R.; [redacted], N.; 2017; M-602452-02-1). Based on the results, the metabolism of BCS-CY26497 (BCS-CN88460-carboxylic acid) in male rats is well understood and the following pathway is depicted:

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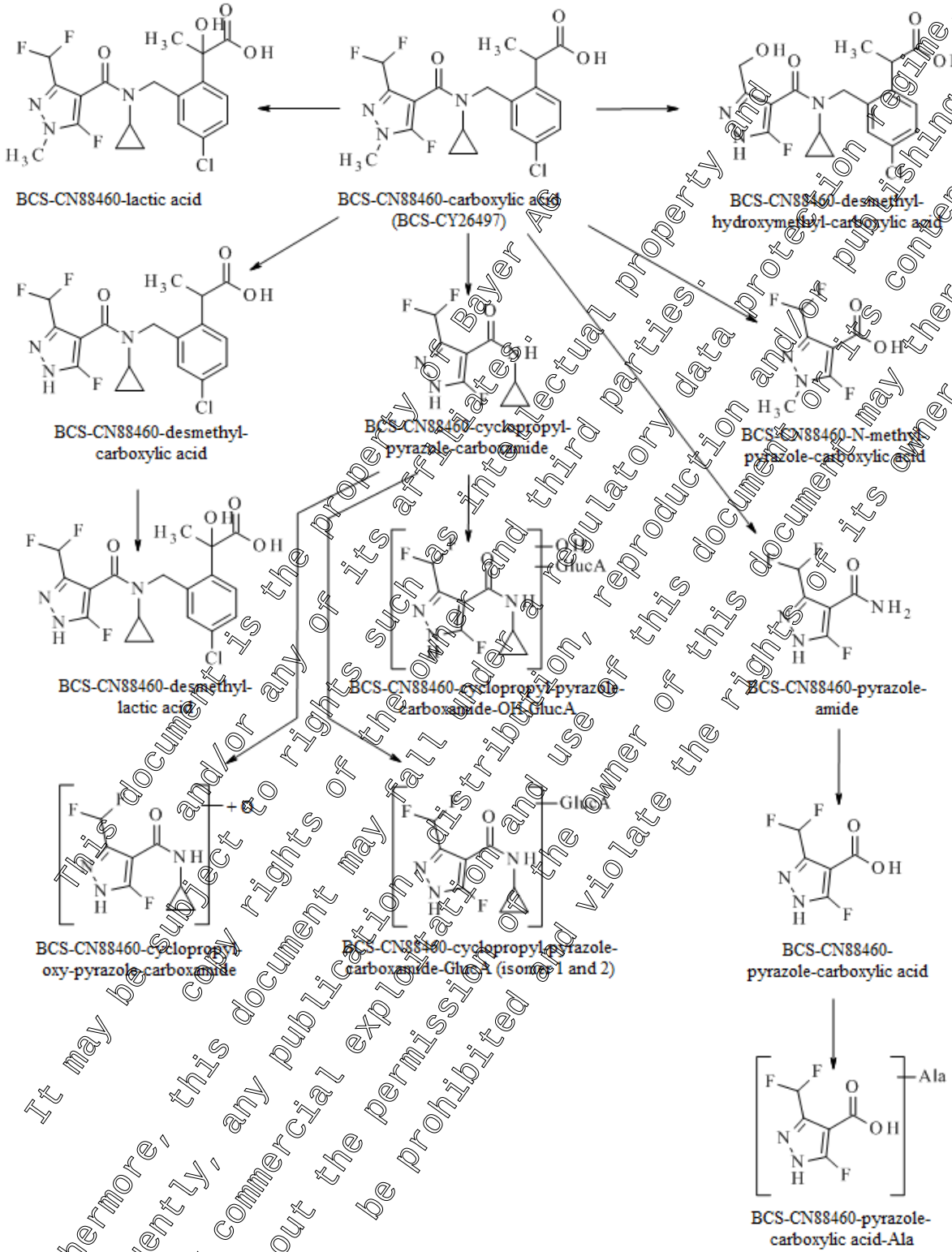
Figure 5.1.1- 4: Proposed metabolic pathway of [pyrazolyl-4-^{14}C]BCS- CY26497 in the rat



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

Type of study	Concentration	Animal species (sex) and test system	Substance	Reference
in-vitro metabolism study	6.6 and 13.2 µM	humans (pool of male & female), rats (male & female), mice (male & female), dogs (male) and rabbits (male), liver microsomes	pyrazole labelled Isoflucypram	M-599295-01-1

Report: KCA 5.1.2/01; [redacted]; 2017; M-599295-01-1
Title: [Pyrazole-4-14C]BCS-CN88460 - Metabolic stability and profiling in liver microsomes from different animals and humans for inter-species comparison
Report No.: EnSa-17-0305
Document No.: M-599295-01-1
Guideline(s): US EPA OCSPP Not Applicable
Guideline deviation(s): none
GLP/GEP: **yes**

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Executive Summary

The *in-vitro* metabolite profile of 6.6 µM and 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 was determined after incubation with liver microsomes from humans (pool of male & female), rats (male & female), mice (male & female), dogs (male) and rabbits (male). The metabolic activity of the enzyme fractions was proven by the metabolisation of the positive control substance ^{14}C-testosterone.

The material balance of each test variant was between 67 and 112% of the applied radioactivity recovered in the supernatants following centrifugation of the incubated suspensions.

As demonstrated with the positive control substance ^{14}C-testosterone the liver microsomes proved to be metabolically active, which was shown by decreasing amount of testosterone and increasing amount of transformation metabolites (up to 15 metabolites). The metabolic transformation rates accounted for up to 96.0% after 30 minutes incubation. Control incubations without NADPH generating biochemical system were analysed by HPLC and demonstrated the stability of the test compound over a time period of 60 minutes.

The *in-vitro* metabolite profile of [pyrazole-4-^{14}C]BCS-CN88460 when incubated with liver microsomes was found to be moderately different in the various microsomal incubations and exhibited moderate to high transformation rates of the applied test compound during the incubation process.

The highest metabolic transformation rate for human liver microsomes was observed after 60 minutes incubation at 13.2 µM accounting for 83.7% of the metabolic transformation. In rat liver microsomes, [pyrazole-4-^{14}C]BCS-CN88460 was metabolised between 62.7% and 98.0% after 60 minutes incubation, with lower metabolic transformation rates for female rat liver microsomes. In mouse liver microsomes, [pyrazole-4-^{14}C]BCS-CN88460 was metabolised between 88.4% and 98.3% after 60 minutes incubation. In the male dog liver microsomes [pyrazole-4-^{14}C]BCS-CN88460 was metabolised up to 95.6% after 60 minutes incubation, and in rabbit liver microsomes up to 95.0%.

Beside BCS-CN88460 up to twenty-one metabolites were detected in the liver microsomes incubates. The metabolic pattern was comparable in human, mouse and rat liver microsomes system and no unique human metabolite was detected. In the various liver microsomes incubates the metabolites 3, 4,

8, 9, 14, 16, 17, 18, 22 and 25 were usually the most abundant metabolites amounting up to 44.69% of the radioactivity. Due to the low amount of radioactivity, all detected metabolites were only characterised based on their chromatographic behavior.

In human liver microsomes the most abundant metabolites 4, 9, 16, 18 and 25 accounted for between 2.89% and 32.20% of the radioactivity.

In rat liver microsomes the most abundant metabolites 3, 4, 8, 14, 16, 17, 18, 22 and 25 accounted for between 0.50% and 44.69% of the radioactivity.

In mouse and dog liver microsomes the most abundant metabolites 4, 14, 16, 17, 18 and 25 accounted for between 1.20% and 30.88% (mouse) or 0.93% and 21.28% (dog) of the radioactivity.

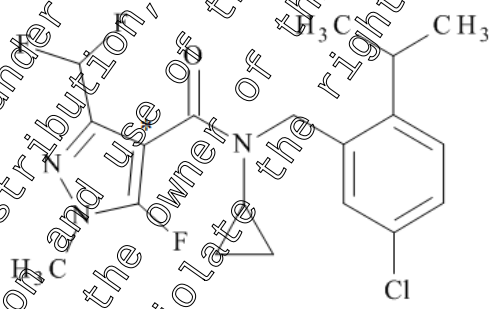
In rabbit liver microsomes the most abundant metabolites 4, 8, 14, 16 and 25 accounted for between 0.58% and 41.30% of the radioactivity.

Other metabolites detected for all liver microsomes incubates accounted for each ≤ 8.62% of the radioactivity.

I. MATERIALS AND METHODS

A. Materials

1. Test material:

Test substance	
Chemical structure	 *denotes the position of the ^{14}C-radiolabel
Radiolabelled test material	[Pyrazole-4-^{14}C]BCS-CN88460
Specific radioactivity	3.90 MBq/mg; equivalent to 233,800 dpm/µg
Radiochemical purity	> 98% (HPLC); >99% (TLC)
Chemical purity	> 99% (HPLC)
Preparation the test solutions	Radiolabelled isoflucypram was dissolved in acetonitrile. Further dilution with acetonitrile and incubation buffer resulted used for the test system at 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460. A 1/10 dilution with the buffer resulted in a second working solution used for the test system at 6.6 µM [pyrazole-4-^{14}C]BCS-CN88460.

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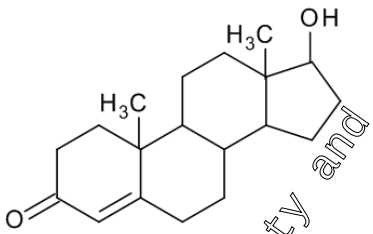
2. Control material (positive control)

Structural formula	 * denotes the position of the ^{14}C-label
Radiolabel	[4-^{14}C]
Specific radioactivity	7.06 MBq/mg
Radiochemical purity	99% (radio-HPLC)
Common name	Testosterone
Chemical name (IUPAC)	(8xi,9xi,14xi,17beta)-17-hydroxyandrost-4-en-3-one
Empirical formula	$C_{19}H_{28}O_2$
Molar mass (unlabelled)	288.41 g/mol
Preparation for administration	Dissolution in acetonitrile and incubation buffer resulted in a control working solution with 10 µM content of [^{14}C-Testosteron

B. Study design

Test system

Liver S9 fractions

Liver microsomes from humans (pool of male & female), Wistar rats (male & female), CD1 mice (male & female), New Zealand rabbits (male), and Beagle dogs (male) were purchased from [redacted]. The liver fractions were stored at -80°C.

The protein concentration was 20 mg protein / mL in each fraction. The cytochrome P450 content was 0.394 nmol/mg protein for the human liver microsomes, 0.703 - 0.780 nmol/mg protein for the rat liver microsomes, 0.789 - 0.939 nmol/mg protein for the mouse liver microsomes, 0.671 nmol/mg protein for the dog liver microsomes and 2.07 nmol/mg protein for the rabbit liver microsomes.

Incubation of [pyrazole-4-^{14}C]BCS-CN88460 with liver microsomes

The incubation system consisted of a phosphate buffer (pH 7.4) and the liver microsomes of humans, rats, mice, dogs and rabbits with a protein concentration of 0.5 mg protein/mL. 6.6 or 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 were incubated separately with the different liver microsomes in the incubation buffer. The enzymatic activity of the incubation was started by addition of the NADPH regeneration system (NADP cofactor containing glucose-6-phosphate dehydrogenase) to the mixture and was stopped after 20, 40 or 60 minutes by addition of 100 µL acetonitrile. All incubations were performed at 37 ± 1°C using a water bath for temperature control. The incubation vessels were gently shaken at a frequency of approx. 120 rpm.

Control incubations were conducted without liver microsomes or without NADPH generating biochemical system in order to show the stability of the test compound in the incubation system. Control incubations were exemplarily performed for 60 minutes at 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460. The [pyrazole-4-^{14}C]BCS-CN88460 working solutions were radioassayed before and after the incubations resulting in a recovery of radioactivity of 67 - 112%.

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Incubation of ^{14}C-testosterone in liver microsomes as positive control

The positive control substance ^{14}C-testosterone (10 µmolar) was incubated with the liver microsomes using a similar composition of the testosterone solution, microsomes and regeneration system as in the test system. These incubations were also conducted at 37°C for a period of 30 minutes.

C. Analytical procedures

Sample processing:

The incubation was stopped by addition of acetonitrile and homogenisation. The resulting suspension was centrifuged for 20 minutes at 16,000 rpm. The supernatant was radioassayed and analysed by radio-HPLC.

Radioassaying (measurement of the radioactivity) of liquid samples was carried out by liquid scintillation counting (LSC) with automatic quench correction.

Radio-HPLC was performed using a RP18 column that was operated with an acidic water/acetonitrile/tetrahydrofurane gradient. The system was equipped with a UV detector (adjusted to 254 nm) and a radioisotope detector.

Evaluation and presentation of the results

The relative composition of the peaks of radio-HPLC analyses were calculated as ratio of the radioactivity of a certain peak by the radioactivity sum of all peaks of the chromatogram.

The metabolic transformation rate of the test compound [pyrazole-4-^{14}C]BCS-CN88460 over time was calculated according to following equation:

$$\% \text{ Metabolic transformation rate} = 100 - \left(\frac{(\% \text{ Area test compound 20, 40 or 60 minutes})}{\% \text{ Area test compound of control incubations after 60 minutes}} \text{ x } 100\ \% \right)$$

where the relative percentages of the peak area of the test compound after 20, 40 and 60 minutes incubation time was compared to the relative percentage measured in the control incubations which were exemplarily performed with liver microsomes incubated at 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460.

II. RESULTS AND DISCUSSION

The *in-vitro* metabolite profile of 6.6 and 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 was determined by HPLC in liver microsomes from mixed humans (pool of male & female), rats (male & female), mice (male & female), rabbits (male) and dogs (male) at 0.5 mg/mL protein concentration. The activity of the enzyme fractions was proven by the metabolisation of the positive control substance ^{14}C-testosterone.

Radioactivity balance

The radioactivity recovered after 20, 40 or 60 minutes was compared to the applied radioactivity. The recoveries were adequate to good in all variants and amounted to 67.3% - 109.5% of the applied radioactivity after 20 minutes, to 84.7% - 111.6% of the applied radioactivity after 40 minutes and to 68.7% - 106.9% of the applied radioactivity after 60 minutes for all incubates of [pyrazole-4-^{14}C]BCS-CN88460 at both concentrations. For the control substance ^{14}C-testosterone the mean recoveries of radioactivity ranged between 121.6% and 126.0% at 30 minutes incubation time.

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Metabolic conversion of the test substance [pyrazole-4-^{14}C]BCS-CN88460

The possible metabolic conversion of the test compound [pyrazole-4-^{14}C]BCS-CN88460 was investigated as 6.6 and 13.2 µmolar solution in liver microsomes from humans, rats, mice, rabbits and dogs. Additionally, control tests were conducted with incubations of [pyrazole-4-^{14}C]BCS-CN88460 in a system without regeneration system (generating NADPH) where no metabolic activity was observed in liver microsomes from humans, rats, mice, rabbits and dogs.

The highest metabolic transformation rate in the human liver microsomes was observed after 60 minutes incubation at 13.2 µM accounting for 83.7% of the metabolic transformation. Transformation rates of 6.6 µM [pyrazole-4-^{14}C]BCS-CN88460 in liver microsomes accounted for up to 51.7% after 40 minutes incubation time and for 34.2% after 60 minutes incubation time

In the male rat liver microsomes the test compound was nearly entirely metabolised (up to 98.0%) after 60 minutes incubation at 6.6 µM and 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460. Transformation rates for female rat liver microsomes after incubation with 6.6µM and 13.2µM accounted for 73.1% and 62.7%, respectively, with slightly higher metabolic transformation rates for female rat liver microsomes after incubation with 6.6 µM [pyrazole-4-^{14}C]BCS-CN88460.

In the male and female mouse liver microsomes the test compound was nearly entirely metabolised (up to 98.3%) after 60 minutes incubation at 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460. Transformation rates for male and female liver microsomes after 60 minutes incubation at 6.6 µM accounted for 92.0% and 88.4%, respectively.

In the male dog liver microsomes the test compound was metabolised up to 95.6% and 92.6% after 60 minutes incubation at 6.6µM and 13.2 µM [pyrazole-4-^{14}C] BCS-CN88460, respectively.

In the male rabbit liver microsomes the test compound was metabolised up to 91.6% and 95.0% after 60 minutes incubation at 6.6µM and 13.2 µM [pyrazole-4-^{14}C] BCS-CN88460, respectively.

Control incubations without NADPH generating biochemical system and without liver microsomes were analysed by HPLC and demonstrated the stability of the test compound over a time period of 60 minutes as the area of the test compound was >99% of the radioactivity.

The results of these incubations are compiled in the table below.

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Table 5.1.2- 1: Metabolic conversion of 6.6 and 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 in liver microsomes

Species/Test system	Relative amount of [pyrazole-4-^{14}C] BCS CN88460 in the radiochromatogram [%]			Rate of conversion*		
Incubation period (minutes)	20	40	60	20	40	60
Human – 6.6 µM						
mixed gender	99.26	47.95	65.23	0	51.7	34.2
Human – 13.2 µM						
mixed gender	39.11	31.40	16.19	60.6	68.3	83.7
control incubation	---	---	99.18	---	---	---
Rat – 6.6 µM						
male	98.93	82.93	3.17	0.3	16.4	96.8
female	52.01	40.97	26.69	47.5	58.7	73.1
Rat – 13.2 µM						
male	7.24	2.98	1.96	92.7	97.0	98.0
control incubation	---	---	99.24	---	---	---
female	57.31	45.01	36.94	42.2	54.6	62.7
control incubation	---	---	99.16	---	---	---
Mouse – 6.6 µM						
male	30.34	5.83	7.94	49.2	94.1	92.0
female	66.94	54.54	11.49	32.5	45.0	88.4
Mouse – 13.2 µM						
male	7.14	1.96	1.82	92.8	98.0	98.2
control incubation	---	---	99.16	---	---	---
female	7.64	8.89	1.65	92.3	91.0	98.3
control incubation	---	---	99.16	---	---	---
Dog – 6.6 µM						
male	87.28	17.92	4.40	11.9	81.9	95.6
Dog – 13.2 µM						
male	39.23	21.57	7.37	60.4	78.2	92.6
control incubation	---	---	99.00	---	---	---
Rabbit – 6.6 µM						
male	57.01	54.74	8.34	42.4	44.7	91.6
Rabbit – 13.2 µM						
male	1.85	2.25	4.95	98.1	97.7	95.0
control incubation	---	---	99.05	---	---	---

* The metabolic transformation rate was calculated on the basis of the % area of [pyrazole-4-^{14}C] BCS-CN88460 in the specific control reactions after incubation of 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 with the different liver microsomes.

Metabolites after Biotransformation of [pyrazole-4-^{14}C]BCS-CN88460

In the various liver microsomes incubates the metabolites 3, 4, 8, 9, 14, 16, 17, 18, 22 and 25 were usually the most abundant metabolites amounting up to 44.69% of the radioactivity. The metabolic pattern in all liver microsomes was qualitatively comparable. Due to the low amount of radioactivity, all detected metabolites were only characterised based on their chromatographic behavior.

Beside parent compound up to twelve metabolites were detected after incubation of 6.6 and 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 with mixed human liver microsomes. All metabolites detected in human liver microsomes were also present in rat microsomes and no unique human metabolite was detected.

Parent compound BCS-CN88460 accounted for between 99.26% and 16.19% of the radioactivity after 20 and 60 minutes incubation, respectively. Metabolites 4, 9, 16, 18 and 25 showed the highest

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abundance and ranged between 2.89% and 32.20% of the radioactivity. All further metabolites detected in human liver microsomes accounted for each ≤ 5.44% of the radioactivity. Metabolites 11, 13, 15, 22 and 24 (each accounting for less than 0.6% of the radioactivity) were only detected in microsomal incubates with 6.6 µM [pyrazole-4-^{14}C] BCS-CN88460.

Beside parent compound up to twenty-one metabolites were detected after incubation of 6.6 and 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 with male and female rat liver microsomes.

Parent compound BCS-CN88460 accounted for between 98.93% and 1.96% of the radioactivity after 20 and 60 minutes incubation, respectively. Metabolites 3, 4, 8, 14, 16, 17, 18, 22 and 25 were the most abundant metabolites detected in male and female rats, whereas the actual amount in male or female rats was different. Their amount ranged thereby between 0.50% and 44.69% of the radioactivity. All further metabolites detected in rat liver microsomes accounted for each ≤ 7.52% of the radioactivity.

The metabolic profiles of the *in-vitro* rat liver microsomes were in good accordance to the metabolite profiles of faeces pool and bile pool samples of male rats after single low dose administration of 2 mg/kg [pyrazole-4-^{14}C]BCS-CN88460. All metabolites detected in rat liver microsomes were also present in the metabolic profiles of faeces and bile pool samples.

Beside parent compound up to twenty-one metabolites were detected after incubation of 6.6 and 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 with male and female mouse liver microsomes.

Parent compound BCS-CN88460 ranged between 66.94% and 1.65% of the radioactivity after 20 and 60 minutes incubation, respectively. Metabolites 4, 14, 16, 17, 18 and 25 showed the highest abundance and ranged between 1.20% and 30.88% of the radioactivity. All further metabolites detected in mouse liver microsomes accounted for each ≤ 7.73% of the radioactivity.

Beside parent compound up to sixteen metabolites were detected after incubation of 6.6 and 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 with male dog liver microsomes.

Parent compound BCS-CN88460 ranged between 87.28% and 4.40% of the radioactivity after 20 and 60 minutes incubation, respectively. Metabolites 4, 14, 16, 17, 18 and 25 showed the highest abundance and ranged between 0.93% and 21.28% of the radioactivity. All further metabolites detected in dog liver microsomes accounted for each ≤ 8.62% of the radioactivity.

Beside parent compound up to thirteen metabolites were detected after incubation of 6.6 and 13.2 µM [pyrazole-4-^{14}C]BCS-CN88460 with male rabbit liver microsomes.

Parent compound BCS-CN88460 ranged between 57.01% and 1.85% of the radioactivity. Metabolites 4, 8, 14, 16 and 25 showed the highest abundance and ranged between 0.58% and 41.30% of the radioactivity. All further metabolites detected in rabbit liver microsomes accounted for each ≤ 6.19% of the radioactivity. In microsomal incubates at 6.6 µM [pyrazole-4-^{14}C]BCS-CN88460 the metabolites showed slightly lower relative abundances.

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Table 5.1.2- 2: Metabolite profile of 6.6 and 13.2 µM [pyrazole-4-14C]BCS-CN88460 in mixed human liver microsomes at 20, 40 and 60 minutes incubation time

Concentration of test item [pyrazole-4-^{14}C] BCS-CN88460	Metabolite	20 min [% of area]	40 min [% of area]	60 min [% of area]
		Human mixed gender		
Human - 6.6 µM	**BCS-CN88460**	**99.26**	**47.93**	**65.23**
	4	---	4.56	2.95
	8	---	---	0.94
	9	---	5.14	5.41
	11	---	---	0.31
	13	---	---	0.24
	14	---	---	1.19
	15	---	---	0.42
	16	---	2.89	3.72
	17	---	3.60	1.73
	18	---	21.07	11.83
	22	---	0.43	---
	24	---	0.53	---
	25	---	8.82	5.83
	26	0.74	5.00	0.69
Human - 13.2 µM	**BCS-CN88460**	**39.11**	**31.40**	**16.19**
	4	6.09	6.59	8.27
	8	---	---	0.77
	9	8.16	9.59	13.23
	14	---	0.70	1.39
	16	3.65	4.04	6.15
	17	4.26	4.90	5.44
	18	24.22	27.54	32.20
	25	9.56	11.49	13.56
	26	4.95	3.74	2.80

--- not detected, below LOQ

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Table 5.1.2- 3: Metabolite profile of 6.6 and 13.2 µM [pyrazole-4-14C]BCS-CN88460 in male and female rat liver microsomes at 20, 40 and 60 minutes incubation time

Concentration of test item [pyrazole-4-^{14}C] BCS-CN88460	Metabolite	20 min [% of area]		40 min [% of area]		60 min [% of area]	
		male	female	male	female	male	female
Rat - 6.6 µM	**BCS-CN88460**	**98.93**	**52.01**	**82.93**	**40.97**	**3.17**	**26.69**
	2	---	---	---	---	0.65	---
	3	---	---	4.96	---	13.85	---
	4	---	0.68	1.89	1.05	9.69	1.38
	6	---	---	---	---	3.16	---
	7a	---	---	---	---	1.04	---
	8	---	---	1.76	0.50	11.64	0.56
	8a	---	---	---	---	0.66	---
	9	---	---	---	0.37	4.16	0.39
	11	---	---	---	---	1.37	---
	12	---	---	0.41	---	1.60	---
	13	---	---	0.77	---	2.24	---
	14	---	1.93	2.30	3.23	16.49	6.49
	15	---	---	0.71	---	2.31	---
	16	---	0.56	2.43	0.91	13.71	1.25
	17	---	8.56	---	9.86	1.83	11.42
	18	---	2.03	1.02	2.48	9.54	2.89
	19	---	---	---	---	1.00	---
	23	---	0.67	---	0.74	---	0.88
	24	---	0.52	---	1.19	0.72	2.22
	25	---	31.33	---	36.90	1.18	44.69
	26	1.07	1.71	0.83	1.79	---	1.15
Rat - 13.2 µM	**BCS-CN88460**	**77.24**	**57.31**	**2.98**	**45.01**	**1.96**	**36.94**
	2	0.41	---	0.60	---	0.57	---
	3	2.78	---	8.59	---	7.66	---
	4	6.91	1.17	10.99	1.40	10.27	1.56
	6	---	---	0.76	---	1.93	---
	7	1.04	---	0.51	---	1.53	0.51
	8	1.19	---	4.96	---	8.64	0.61
	9	4.10	---	5.16	---	7.45	0.77
	11	0.66	---	1.64	---	1.32	---
	12	---	---	---	---	0.44	---
	13	1.27	---	1.38	---	1.40	---
	14	4.51	1.78	11.44	2.60	12.17	4.40
	15	---	---	1.36	---	1.64	---
	16	5.70	---	9.86	1.00	9.98	1.44
	17	18.57	9.58	8.93	10.54	9.24	12.42
	18	20.74	2.17	16.93	3.47	17.61	2.92
	20	3.90	---	7.52	0.46	1.54	---
	22	11.66	---	3.45	0.86	0.70	---
	23	0.60	---	---	0.74	---	0.69
	24	0.33	0.31	---	0.49	0.51	1.33
	25	7.61	25.68	2.95	30.86	3.44	34.07
	26	0.79	2.00	---	2.57	---	2.32

--- not detected, below LOQ

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Table 5.1.2- 4: Metabolite profile of 6.6 and 13.2 µM [pyrazole-4-14C]BCS-CN88460 in male and female mouse liver microsomes at 20, 40 and 60 minutes incubation time

Concentration of test item [pyrazole-4-^{14}C] BCS-CN88460	Metabolite	20 min [% of area]		40 min [% of area]		60 min [% of area]	
		male	female	male	female	male	female
Mouse - 6.6 µM	**BCS-CN88460**	**50.34**	**66.94**	**5.83**	**54.54**	**7.94**	**11.49**
	1	0.74	---	1.17	---	1.69	---
	3	4.04	3.38	6.30	6.23	7.46	7.55
	4	8.34	6.23	14.42	9.41	14.87	16.75
	6	0.83	0.84	1.49	---	1.13	1.17
	7	---	---	---	---	0.38	---
	8	2.75	1.52	5.99	1.59	7.73	3.18
	8a	1.01	---	2.02	0.52	3.28	---
	9	1.27	---	2.44	1.05	2.53	1.04
	13	2.70	2.01	4.07	4.29	4.38	3.75
	14	13.78	5.81	30.88	7.36	28.79	18.17
	15	---	---	1.24	0.45	0.98	---
	16	6.11	4.55	11.97	5.03	11.75	10.42
	17	1.97	2.32	2.11	1.20	3.42	6.47
	18	2.98	3.69	4.98	1.79	3.31	9.40
	19	1.16	0.68	1.68	0.79	0.94	---
	20	---	---	---	1.80	---	2.69
	21	---	---	0.56	---	---	0.52
	23	---	---	0.32	---	---	0.69
	24	---	---	0.27	---	---	0.53
	25	1.46	1.51	2.16	1.27	1.43	5.95
	26	0.54	0.51	---	0.68	---	0.23
Mouse - 13.2 µM	**BCS-CN88460**	**7.14**	**7.64**	**1.96**	**8.89**	**1.82**	**1.65**
	1	---	---	0.68	0.84	1.38	1.75
	3	6.40	6.25	6.58	4.99	7.18	7.50
	4	15.61	20.73	15.40	23.00	15.90	21.98
	6	1.76	2.01	1.61	1.29	1.61	1.94
	8	3.04	3.87	4.32	3.74	5.83	5.20
	8a	---	---	0.96	---	1.53	1.03
	9	1.09	0.60	1.96	1.35	2.20	2.02
	13	2.21	2.62	2.81	3.74	3.79	4.89
	14	19.89	17.03	23.82	23.06	29.37	24.06
	15	---	---	---	---	0.77	1.13
	16	8.36	10.42	10.31	13.30	12.10	14.44
	17	10.72	6.79	7.85	2.08	4.93	2.43
	18	10.41	11.04	9.03	7.40	7.37	6.44
	19	---	1.18	1.66	2.49	---	1.31
	20	---	---	1.09	---	---	---
	21	1.30	1.08	1.82	---	1.01	0.45
	23	1.34	1.15	0.98	---	---	---
	25	10.73	7.59	7.15	3.82	3.20	1.77

--- not detected, below LOQ

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Table 5.1.2- 5: Metabolite profile of 6.6 and 13.2 µM [pyrazole-4-14C]BCS-CN88460 in male dog liver microsomes at 20, 40 and 60 minutes incubation time

Concentration of test item [pyrazole-4-^{14}C] BCS-CN88460	Metabolite	20 min [% of area]	40 min [% of area]	60 min [% of area]
		male	male	male
Dog - 6.6 µM	**BCS-CN88460**	**87.28**	**17.92**	**4.40**
	3	---	0.44	0.71
	4	0.93	7.83	10.29
	8	0.21	1.07	1.68
	8a	---	0.47	0.73
	9	0.90	7.20	8.62
	11	---	0.35	0.49
	13	---	0.67	1.49
	14	2.07	9.39	15.33
	16	1.21	7.49	11.06
	17	1.32	9.87	9.63
	18	3.03	19.01	20.82
	21	---	2.10	2.04
	22	---	0.40	---
	24	---	2.32	2.54
	25	2.29	13.11	9.46
	26	0.75	0.39	0.73
Dog - 13.2 µM	**BCS-CN88460**	**39.23**	**21.57**	**7.37**
	3	---	0.50	1.17
	4	7.55	10.55	10.27
	7	---	0.88	---
	8	---	0.45	1.17
	9	4.24	5.98	8.26
	14	2.69	5.10	12.95
	16	3.11	5.23	8.76
	17	9.03	10.84	11.35
	18	15.77	20.00	21.28
	21	---	1.31	2.40
	24	---	0.90	1.95
	25	16.19	15.61	13.07
	26	2.19	1.07	---

--- not detected, below LOQ

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Table 5.1.2- 6: Metabolite profile of 6.6 and 13.2 µM [pyrazole-4-14C]BCS-CN88460 in male rabbit liver microsomes at 20, 40 and 60 minutes incubation time

Concentration of test item [pyrazole-4-^{14}C] BCS-CN88460	Metabolite	20 min [% of area]	40 min [% of area]	60 min [% of area]
		male	male	male
Rabbit - 6.6 µM	**BCS-CN88460**	**57.01**	**54.74**	**8.34**
	3	0.30	0.75	1.39
	4	2.86	3.30	7.22
	8	6.90	7.21	13.82
	9	0.73	---	1.67
	13	1.00	1.38	2.50
	14	10.69	12.43	23.23
	16	15.76	17.48	39.45
	17	1.73	---	---
	18	0.88	---	---
	25	2.58	0.58	2.38
	26	0.56	0.54	---
	28	---	0.59	---
Rabbit - 13.2 µM	**BCS-CN88460**	**1.85**	**2.25**	**4.95**
	2	0.27	0.44	0.92
	3	1.52	0.71	1.56
	4	9.54	9.01	8.53
	8	14.45	10.37	16.29
	9	1.61	4.16	---
	13	1.70	1.23	2.08
	14	17.87	10.51	20.95
	15	0.82	0.53	1.07
	16	41.30	36.78	39.54
	17	2.48	6.19	1.81
	18	0.45	5.37	---
	23	0.47	---	---
	25	5.68	12.45	2.31

--- not detected, below LOQ

Metabolic conversion of the positive control ^{14}C-testosterone

Liver microsomes were metabolically active as demonstrated by the metabolic conversion of ^{14}C testosterone. Decreasing amounts of testosterone with increasing formation of radioactive metabolites (up to 15) demonstrated sufficient metabolic capability of the liver microsomes used in this study. The metabolic activities of human, rat, mouse and rabbit liver microsomes ranged between 5.8% and 96.0% of the metabolic activity. Rat liver microsomes showed the highest metabolic activity with 71.4% and 96.0% activity for female and male rat liver microsomes, respectively. Male rabbit liver microsomes had a metabolic activity of 23.5%. Female and male mouse liver microsomes had a metabolic activity of 7.2% and 8.1%, respectively. Human liver microsomes showed the lowest metabolic activity of 5.8%. Dog liver microsomes showed no metabolic activity in this test, whereas the metabolic transformation rate of the test compound [pyrazole-4-^{14}C]BCS-CN88460 amounted up to 96% in male dog liver microsomes.

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III. CONCLUSIONS

[Pyrazole-4-^{14}C]BCS-CN88460 was incubated with liver microsomes from humans (pool of male & female), rats (male & female), mice (male & female), dogs (male) and rabbits (male) for 20, 40 and 60 minutes at 37°C. The biotransformation rate of radiolabelled [pyrazole-4-^{14}C]BCS-CN88460 and the metabolic pattern was found to be moderately different in the various microsomal incubations. The enzymatic activity of each of the liver microsomes was demonstrated by a significant metabolic conversion of the positive control substance ^{14}C-testosterone. Beside BCS-CN88460 up to twenty-one metabolites were detected in the liver microsomes incubates after biotransformation of [pyrazole-4-^{14}C]BCS-CN88460. A higher number of metabolites were detected in liver microsomes from rat, mouse and dog, whereas a lower number of metabolites were detected in liver microsomes from rabbit and humans. In the various liver microsomes incubates the metabolites 3, 4, 8, 9, 14, 16, 17, 18, 22 and 25 were usually the most abundant metabolites. The metabolic pattern in all liver microsomes was qualitatively comparable. All metabolites formed by microsomes from humans were also detectable in microsomes from rat and no human unique metabolites were detected. The metabolic profiles of the *in-vitro* rat liver microsomes were in good accordance to the metabolite profiles of faeces pool and bile pool samples of male rats after single low dose administration of 2 mg/kg [pyrazole-4-^{14}C]BCS-CN88460 ([redacted], R.; [redacted], N.; 2017; M-602452-02-1). Due to the low amount of radioactivity, all detected metabolites were only characterised based on their chromatographic behavior.

CA 5.2 Acute toxicity

Table 5.2-1: Summary of Isoflucypram acute toxicity data, with classification endpoints according to Regulation (EC) No 1272/2008

Study	Result	Reference	Classification according to Reg. (EC) No 1272/2008
Acute oral rat	Oral LD50 > 2000 mg/kg bw	[redacted]; 2014; M-485872-01-1	Category 5 / unclassified
Acute dermal rat	Dermal LD50 > 2000 mg/kg bw	[redacted]; 2014; M-485659-01-1	Category 5 / unclassified
Acute inhalation rat	Inhalation LC50 = 2.518 mg/L (both sexes combined) 3.131 mg/L (males) 2.209 mg/L (females)	[redacted]; 2014; M-502440-01-1	Category 4, H332 Harmful if inhaled
Skin irritation, rabbit	Negative	[redacted]; 2014; M-484711-01-1	Category 5 / unclassified
Eye irritation *in vitro*, isolated chicken eyes	Neither severe irritant nor non-irritant	[redacted]; 2014; M-488523-01-1	Inconclusive
Eye irritation, rabbit	Negative	[redacted]; 2014; M-493768-01-1	Category 5 / unclassified
Skin sensitization (LLNA)	Sensitizing EC3 = 29.0%	[redacted]; 2015; M-524452-01-1	Category 1B, H317 May cause an allergic skin reaction
Phototoxicity	Negative	[redacted], C.; 2018; M-613506-02-1	unclassified

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All *in vivo* acute toxicity studies were conducted between 2014 and 2015 and were fully compliant with Good Laboratory Practice (GLP) standards.

The acute toxicity of BCS-CN88460 was low for oral and dermal routes of exposure. In the rat acute oral study, there were no mortalities, systemic clinical signs, alterations in body weight or body weight gain, or macroscopic findings. The oral LD_{50} was > 2000 mg/kg bw, the highest dose tested. In the rat acute dermal study, there were no mortalities, systemic clinical signs, alterations in body weight or body weight gain, or macroscopic findings at necropsy. Very slight erythema was noted in several animals on the first day of the study only. The dermal LD_{50} was > 2000 mg/kg bw, the highest dose tested.

In the rat acute inhalation study, some mortalities were noted at higher doses during the 4-hour nose-only exposure period. Clinical signs in animals which survived the exposure period included noisy or gasping respiration, decreased activity, slight to severe ataxia, hunched back, slight sneezing, prostration, or coma. All surviving animals were symptom-free by study day 3 at the latest. In animals found dead, dark or red diffuse or multifocal discoloration of the lungs were observed. No macroscopic findings were noted at necropsy of surviving animals. The inhalation LC_{50} value following a 4-hour, nose-only exposure period was 2.518 mg/L when both sexes were combined, or 3.131 mg/L in males and 2.209 mg/L in females. This value triggers classification under Regulation (EC) 1272/2008 in Category 4 for acute inhalation toxicity.

After dermal application to the shorn skin of rabbits, there were no signs of irritation. After instillation of 0.1g to the conjunctival sac of the eye, conjunctival redness, chemosis, and discharge were noted in all three treated animals. The chemosis and discharge were reversible within 48 hours of application, while the conjunctival redness was reversible after 72 hours. No corneal effects were observed in any animal.

In the mouse local lymph node assay, ear thickness was not increased in a biologically meaningful way, and there were no visible signs of an irritant effect up to the highest tested concentration of 50%. The Stimulation Indices (SI) are 5.6, 2.5, 1.2, and 1.2 at concentrations of 50%, 25%, 10%, and 5%. As the values at 25% and 50% exceeded the SI trigger value of 3, the compound is concluded to be a skin sensitizer in the mouse local lymph node assay. This triggers classification under GHS (rev. 5, 2013) as Skin Sens. Category 1B, H317.

The *in vitro* phototoxicity study was negative, and thus isoflucypram is not phototoxic. The *in vitro* photomutagenicity study is not triggered.

CA 5.2.1 Oral

Report:	KCA 5.2.1/01; [redacted], 2014; M-485872-01-1
Title:	BCS-CN88460 technical - Acute oral toxicity study in the rat (up and down procedure)
Report No.:	14/069-001R
Document No	M-485872-01-1
Guideline(s):	OECD No.: 425, adopted October 2008 EC NO 440/2008 OF 30 MAY 2008, B.1.TRIS EPA OPPTS 870.1100, EPA 712-C-02-190, December 2002
Guideline deviation(s):	none
GLP/GEP:	**yes**

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Executive Summary

An acute oral toxicity study was conducted in the female rat with BCS-CN88460. After overnight diet fasting, 5 female rats were sequentially administered a single dose of BCS-CN88460 by oral gavage at a dose level of 2000 mg/kg bw/day, followed by a 14-day observation period. Diet was returned 3 hours after dosing. All animals were observed individually after dosing at 30 minutes and 1, 2, 3, 4, and 6 hours after treatment, and once each day for 14 days thereafter. Body weight was measured the day before and the day of dosing and weekly thereafter. At the end of the observation period, animals were necropsied.

No mortalities, clinical signs, effects on body weight or body weight gain, or macroscopic findings were observed in any animal following oral gavage administration of BCS-CN88460 to female rats. The oral LD50 of BCS-CN88460 is therefore greater than 2000 mg/kg bw in the female rat. This triggers classification and labelling under GHS (rev. 7, 2017) of Category 5 / unclassified; H303 (May be harmful if swallowed).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460 technical
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2%, w/w
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

2. Vehicle and / or positive control: PEG 400

3. Test animals:	
Species:	Rat
Strain:	RccHan:(WIST)
Age:	9-11 weeks old
Weight at dosing:	180-193g
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	[redacted]® Sm R/M autoclavable complete diet for rats and mice, ad libitum
Water:	Tap water from municipal supply, ad libitum
Housing:	Individual housing in polypropylene / polycarbonate cages, with deep Lignocell bedding
Environmental conditions:	
Temperature:	22 ± 3°C
Humidity:	30-70%
Air changes:	15-20 per hour
Photoperiod:	12hours daily

B. STUDY DESIGN AND METHODS

1. In life dates: 26 February-25 March 2014

2. Animal assignment and treatment

The assignment of animals to groups is not relevant for a study in which only one group of animals is treated. Animals were individually identified by numbers written on the tail with a permanent marker pen.

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The test compound was suspended in PEG 400 at a concentration of 200 mg/ml on the day of administration, and was stirred with a magnetic stirrer until the treatment was finished.

A limit test of 2000 mg/kg bw was conducted, using a dose volume of 10 ml/kg bw of the dose solution. A single oral gavage administration was followed by a 14-day observation period. The animals were fasted overnight prior to treatment, although water was still available ad libitum overnight. Animals were weighed before dosing, and food was returned 3 hours after treatment.

Single animals were dosed sequentially, with intervals of at least 48 hours between animals. These time intervals were determined by the onset, duration, and severity of toxic signs.

All animals were observed individually at 30 minutes, after dosing, then after 1, 2, 3, 4 and 5 hours after dosing, and once each day for 14 days thereafter. Individual observations were performed on the skin and fur, eyes, and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity, and behavior pattern. Body weights were recorded on the day prior to treatment, on the day of administration (study day 0) before dosing, and on study days 7 and 14.

Animals were sacrificed by exsanguination under pentobarbital anesthesia. After examination of the external appearance, the cranial, thoracic, and abdominal cavities were opened and macroscopic abnormalities were recorded.

3. Statistics

The Acute Oral Toxicity (OECD Test Guidelines 425) Statistical Programme (AOT 425 Stat Pgm) was used to estimate the oral LD50.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities after oral gavage administration of BCS-CN88460.

B. CLINICAL OBSERVATIONS

No clinical signs were observed in any animal at any time point.

C. BODY WEIGHT

Body weight and body weight gain of animals administered BCS-CN88460 by oral gavage was not affected by treatment.

D. NECROPSY

There were no macroscopic observations at 2000 mg/kg bw.

E. DEFICIENCIES

No deficiencies are noted.

III. CONCLUSIONS

Under the conditions of this study, the acute oral LD50 value of BCS-CN88460 was greater than 2000 mg/kg bw/day in the female rat. This result does not trigger classification or labelling under EU Directive 1999/45/EC (as amended), Regulation (EC) No 1272/2008 (CLP). Under GHS (rev. 7, 2017), the following classification and labelling is triggered: Category 5 / unclassified; H303 (May be harmful if swallowed).

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CA 5.2.2 Dermal

Report:	KCA 5.2.2/01; [redacted]; 2014; M-485659-01-1
Title:	BCS-CN88460 technical - Acute dermal toxicity study in rats.
Report No.:	14/069-002P
Document No.:	M-485659-01-1
Guideline(s):	OECD 402 (1987); EPA OPPTS 870.1200 (1998); EC 440/2008 (2008)
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Executive Summary

The acute dermal toxicity of BCS-CN88460 was determined through a single dermal administration of the test item for 24 hours under a semi-occlusive bandage, followed by an observation period of 14 days. The test item was applied dermally to five male and five female Wistar rats, using a limit dose of 2000 mg/kg bw/day in both sexes.

Clinical observations were performed on all animals at 1 and 5 hours after dosing and daily for 14 days thereafter. Body weight was measured prior to compound application on study day 0 and on study days 7 and 14. Rats were euthanized and subjected to a gross macroscopic examination on study day 14.

There were no mortalities, systemic clinical signs, or treatment-related effects on body weight or body weight gain, nor were there any abnormalities noted at necropsy. Very slight erythema (score 1) was noted in five of 10 animals (2 males and 3 females) on study day 1 only. All local signs observed were reversible by study day 2.

The median lethal dose of BCS-CN88460 after a single dermal administration in the male and female Wistar rat was determined to be greater than 2000 mg/kg bw. The study result therefore triggers classification under GHS (rev. 7, 2017) as Category 5 / unclassified.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2%, w/w
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

2. Vehicle and / or positive control: no vehicle was used; test compound was applied to gauze pad moistened with water.

3. Test animals:	
Species:	Rat
Strain:	RccHan:(WIST)
Age:	Only described as young adult
Weight at dosing:	204-246g
Source:	[redacted]
Acclimation period:	7 days
Diet:	[redacted]® Sm R/M autoclavable complete diet for rats and mice,

	ad libitum
Water:	Tap water from municipal supply, ad libitum
Housing:	Individual housing in polypropylene / polycarbonate cages, with deep Lignocell bedding
Environmental conditions:	
Temperature:	22 ± 3°C
Humidity:	30-70%
Air changes:	15-20 per hour
Photoperiod:	12 hours daily

B. STUDY DESIGN AND METHODS

1. **In life dates:** 19 February-12 March 2014

2. **Animal assignment and treatment**
As only one group per sex was treated, there was no need for specific assignment to groups.

An area on the back of each animal, measuring approximately 10% of the total body surface area, was shorn approximately 24 hours prior to test item application. The application site was inspected prior to treatment and only those animals without injury or irritation of the skin were used.

On study day 0, the test item was applied to a moistened gauze pad at an amount to ensure a dose of 2000 mg/kg bw/day for each animal. The gauze pad with test item was placed on the application site and secured by a patch with adhesive hypoallergenic plaster, then the entire trunk of the animal was wrapped with semi-occlusive plastic wrap for 24 hours. At the end of the exposure period, any residual test item was removed using body temperature water.

Clinical examinations were performed on the day of treatment at 1 and 5 hours after the application of the test item, and once each day for 14 days thereafter. Observations included the skin and fur, eyes and mucus membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern.

The body weight of all animals was recorded on study days 0, 7, and 14.

On study day 14, animals were exsanguinated under pentobarbital anesthesia. The external appearance was evaluated, the cranial, thoracic, and abdominal cavities were examined, and any macroscopic findings were noted.

3. **Statistics**
Specific statistical methods were not used.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities in either males or females after dermal administration of BCS-CN88460.

B. CLINICAL OBSERVATIONS

No clinical signs were observed in any animals during the study.

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C. LOCAL DERMAL SIGNS

Local signs including very slight erythema (score 1) were noted in two males and three females on study day 1 (the day following test item application); all animals were free from local dermal findings on study day 2 and for the remainder of the observation period.

D. BODY WEIGHT

There were no treatment-related effects on body weight or body weight gain during the observation period.

E. NECROPSY

There were no macroscopic findings noted in any animal at necropsy.

F. DEFICIENCIES

No deficiencies are noted.

III. CONCLUSIONS

Under the circumstances of this study, the medial lethal dose of BCS-CN88460 after a single dermal administration was found to be greater than 2000 mg/kg bw in male and female Rcc:Han: WIST rats. The study does not trigger any classification or labelling under Regulation (EC) No 1272/2008 (CLP). Under GHS (rev. 7, 2017), classification for acute dermal toxicity of Category 5 / unclassified is appropriate.

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CA 5.2.3 Inhalation

Report:	KCA 5.2.3/01; [redacted]; 2014; M-502440-01-1
Title:	BCS-CN88460 technical - Acute inhalation toxicity study (nose-only) in the rat
Report No.:	14069-004P
Document No.:	M-502440-01-1
Guideline(s):	OECD Test Guideline 403 (2009); EPA OPPTS 870.1300 (1998); EC 440/2008, Annex Part B, B.2 (2008)
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Executive Summary

Male and female Wistar rats were exposed to a liquid aerosol atmosphere of BCS-CN88460 (diluted to 10-20% w/w with acetone then aerosolized) at concentrations of up to 2.87 mg/L for four hours.

Clinical observations were performed for all animals during exposure at hourly intervals, following removal from restraint, approximately 1 hour following the end of exposure, and daily for 14 days thereafter. Body weight was measured on study day 0 before the exposure, and on study days 1, 3, 7, and 14 or at death. Gross necropsy was performed on all animals sacrificed on study day 14.
The MMAD in the various exposure groups ranged from 1.41 to 1.81 µm, with GSD from 1.81 to 1.99.
One male and one female died at 2.03 mg/L, while two males and all five females died at 2.87 mg/L during the exposure. Clinical signs observed in animals which survived the exposure period included noisy or gasping respiration, decreased activity, ataxia (slight to severe), hunched back, slight sneezing, prostration, or coma. All surviving animals were symptom-free by study day 3 at the latest.

Dark or red diffuse or multifocal discoloration of the lungs were observed in all animals which were found dead. Among surviving animals, there were no findings of note at necropsy.

Under the experimental conditions of this study, the 4-hour acute inhalation medial lethal concentration (LC50) of BCS-CN88460 in the Wistar rat is as follows:

- 2.518 mg/L, combined for both males and females (95% confidence limits: 2.016-3.663 mg/L)
- 3.131 mg/L for males (95% confidence limits not calculated as their range is too wide)
- 2.209 mg/L for females (95% confidence limits not calculated as their range is too wide)

The study result triggers the following classification and labelling:

- EU Directive 1999/45/EC: Xn, harmful
- Regulation (EC) No 1272/2008 (CLP): Category 4
- GHS (rev. 7, 2017): Category 4

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2%, w/w
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

2. Vehicle and / or positive control: acetone

3. Test animals:	
Species:	Rat
Strain:	Wistar Crl:WI
Age:	Sighting study, approximately 11 weeks Main study, approximately 8-9 weeks
Weight at dosing:	Sighting study: 402-420g (male) or 241-242g (female) Main study: 284-475g (male) or 193-263g (female)
Source:	[redacted]
Acclimation period:	28-35 days (sighting study) or 8-15 days (main study)
Diet:	[redacted] SM R/M autoclavable complete feed for rats and mice
Water:	Tap water ad libitum
Housing:	Singly (sighting study) or by groups of 5 (main study) in polycarbonate solid floor cages with stainless steel mesh lids with deep Lignocel bedding
Environmental conditions:	
Temperature:	17.0-25.0°C
Humidity:	32-70%
Air changes:	At least 15 air exchanges per hour
Photoperiod:	12 hours of light, 12 hours of dark

B. STUDY DESIGN AND METHODS

1. In life dates: 10 April-27 June 2014

2. Animal assignment and treatment

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Animals were randomly allocated to exposure groups based on actual body weights. After allocation, animals were individually identified with numbers written on the tail with an indelible marker.

The animals were exposed to an atmosphere of the test item formulation for a single, continuous four-hour period or until total death of the group. The study was conducted according to the following design.

Group number	Target conc. (mg/L)	Mean achieved conc. (mg/L)	Duration of exposure (h)	Number of animals	
				Male	Female
Sighting exposure					
0.1	5	4.97	4	1	1
0.2	1	0.97	4	1	1
Main study					
1	1	1.03	4	5	5
2	3	2.82	4	5	5
3	2	2.04	4	5	5

3. Generation of the test atmosphere / chamber description

Prior to animal exposures, test material atmospheres were generated within the exposure chamber. During these technical trials, air-flow settings, test material input rats, and test item formulation concentrations (10-100%) were varied to achieve the required aerosol concentration of particles with a mass median aerodynamic diameter (MMAD) between 1 and 4 µm and a geometric standard deviation (GSD) in the range of 1.5 to 3.0. Measurements of aerodynamic particle size were performed within the breathing zone using a cascade impactor.

The test item was diluted with acetone to 10% or 20% w/w concentration depending on the target concentrations. Before nebulization, the formulation was homogenized using a magnetic stirrer. This procedure was considered not to alter the chemical composition of the test item. The test item was aerosolized using a stainless steel concentric jet nebulizer at the top of the exposure chamber with the rate of test item usage controlled by a syringe pump.

The animals were exposed, nose-only, to an atmosphere of the test item using a TSE rodent exposure system comprising two concentric anodized aluminium chambers and a computer control system incorporating pressure detectors and mass flow controllers. Fresh aerosol from the generation system was constantly supplied to the inner plenum distribution chamber) of the exposure system from where, under positive pressure, it was distributed to the individual exposure ports. The animals were held in polycarbonate restraint tubes located around the chamber which allowed only the animal's nostrils to enter the exposure port. After passing through the animal's breathing zone, used aerosol entered the outer cylinder from where it was exhausted through a suitable filter system.
Airflows and relative pressures within the system were constantly monitored and controlled by the computer system thus ensuring a uniform distribution and constant flow of fresh aerosol to each exposure port. The flow of air through each port was at least 0.5L/min, which was considered adequate to minimize re-breathing of the test atmosphere as it is approximately twice the respiratory minute volume of a rat.

Following an equilibration period of at least the theoretical chamber equilibration time, each group of rats was exposed to an atmosphere of the test material for a period of 4 hours or until the death of all animals, whichever came earlier.

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The test atmosphere was sampled at regular intervals during the exposure period. Samples were taken from an unoccupied exposure port (representing the animal's breathing zone) by pulling a suitable volume of test atmosphere through weight GF10 glass fiber filters. The difference in the pre- and post-sampling (dried) weights divided by the volume of atmosphere sampled was equal to the actual achieved test atmosphere concentration. Filter samples were collected at the breathing zone during each 4-hour exposure period and analyzed.

The particle size of the test atmosphere was determined three times during the exposure period using a 7-stage impactor of Mercer style. Samples were taken from an unoccupied exposure port, and the collection substrates and the backup filter were weighed before and after the sampling, and the weight of test item collected at each stage was calculated. The total amount collected for each stage was used to determine the cumulative amount below each cut-off point, and in this way the proportion of aerosol < 0.550, 0.550, 0.960, 1.550, 3.444, 6.655, and 10.550 µm was calculated. The MMAD and GSD were then calculated, and the proportion of aerosol less than 4 µm was determined.

Animals were checked hourly during exposure, 1 hour after exposure, and twice daily during the 14-day observation period for morbidity and / or mortality. All animals were observed for clinical signs at hourly intervals during exposure while the animals were still restrained. Following exposure, clinical observations were performed twice on the day of exposure and subsequently once daily for 14 days.

Individual body weights were recorded prior to treatment on the day of exposure and on study days 1, 3, 7, and 14, or at death.

At the end of the 14-day observation period, surviving animals were sacrificed by exsanguination under pentobarbital anesthesia. All animals regardless of the day of death were subject to a gross necropsy which included a detailed examination of the abdominal and thoracic cavities. Special attention was given to the respiratory tract for macroscopic signs of irritancy or local toxicity.

4. Statistics

The four-hour inhalation LC50 and 95% confidence limits were calculated by probit analysis using SPSS PC+4.0 software, and were calculated for males and females separately and as a combined value for both sexes.

II. RESULTS AND DISCUSSION

A. TEST ATMOSPHERE CONCENTRATION AND PARTICLE SIZE ANALYSIS

The test atmospheres were sampled from the breathing zone during each exposure period, 4-18 times at approximately equal intervals. Analysis of the particle size distribution of the aerosol at the animals' breathing zone demonstrated that the test atmosphere was respirable. The mean achieved actual concentrations and nominal concentrations, and the mean values of the particle size distribution parameters calculated from two or three samples of each exposure are presented in Table 5.2.3/01-1.

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Table 5.2.3/01-1: Mean achieved actual and nominal aerosol concentration, MMAD, GSD, and inhalable fraction (% < 4 µm) for each treatment group

	BCS-CN88460 treatment group			4 (acetone control)
Parameter	**1**	**2**	**3**	
Target conc., mg/L	1	3	2	0
Mean achieved conc., mg/L	1.03	2.87	2.04	0.0
SD of achieved conc., mg/L	0.07	0.14	0.14	0.0
Nominal conc., mg/L	3.27	9.36	6.52	84.45 (acetone)
MMAD, µm	1.52	1.41	1.56	
GSD	1.99	1.93	1.88	
Inhalable fraction (% < 4 µm)	92.0	94.3	93.2	

B. MORTALITY

In group 2, at a concentration of 2.87 mg/L, two males and all females died during exposure. In group 3, at a concentration of 2.04 mg/L, one male and one female died during exposure.

C. CLINICAL OBSERVATIONS

At a concentration of 1.03 mg/L, clinical observations were confined to the day of exposure, and included laboured respiration (slight to moderate) in all rats, and in two males noisy respiration (slight) and gasping respiration in two males. Decreased activity (slight) in all animals, ataxia (slight) in two males and all females, and hunched back in a single female rat were also observed on the day of exposure.

In group 2, at a concentration of 2.87 mg/L, laboured respiration (moderate to severe) was recorded in all rats on day 0 and up to day 1. Noisy respiration (slight to severe) in three males on day 1 or day 2, gasping respiration in one male on day 1 and slight sneezing in one male on day 1 were also noted in this group. The three surviving males were prostrated after the exposure on day 0.

All surviving rats at 2.04 mg/L showed laboured respiration (slight to severe) on days 0-1 and gasping respiration in all surviving rats on the day of exposure. Noisy respiration (slight) was also recorded in one male on day 1. In addition, severe ataxia and moderately decreased activity were seen in a single surviving male on day 0. Two males were prostrated and all surviving rats were comatose after the exposure on day 0.

In the acetone control group, all animals had moderately laboured respiration and were prostrated after the exposure on day 0.

D. BODY WEIGHT

Slight body weight loss or body weight retardation was noted in all groups on day 1. All surviving animals had returned to their initial body weights no later than study day 7.

E. NECROPSY

Dark / red diffuse or multifocal discoloration of the non-collapsed lungs and / or light brown liquid material at the perioral / perinasal fur were observed at necropsy in all animals which were found dead. Neither external nor internal findings were recorded in the surviving study animals at necropsy.

F. DEFICIENCIES

No specific deficiencies were noted during the study.

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III. CONCLUSIONS

Under the experimental conditions of this study, the 4-hour acute inhalation medial lethal concentration (LC50) of BCS-CN88460 in the Wistar rat is as follows:

- 2.518 mg/L, combined for both males and females (95% confidence limits: 2.010-3.663 mg/L)
- 3.131 mg/L for males (95% confidence limits not calculated as their range is too wide)
- 2.209 mg/L for females (95% confidence limits not calculated as their range is too wide)

The study result triggers the following classification and labelling:

- EU directive 1999/45/EC: Xn, harmful
- Regulation (EC) No 1272/2008 (CLP): Category 4
- GHS (rev. 7, 2017): Category 4

CA 5.2.4 Skin irritation

In vitro skin corrosion or irritation studies were not conducted prior to the conduct of this *in vivo* skin irritation study. As no indications of corrosion or pain reactions were observed in the dermal toxicity study conducted in the rat (KCA 5.2.2/01, M-485659-01-1), and as the pH of the test substance dissolved in water was measured by the conducting laboratory to be 4.5, it was considered that no conditions existed which would prevent the conduct of the *in vivo* study. The possibility for pain and distress to the animals was minimized by only treating one animal initially, followed by treatment of the remaining two animals once it was clear that dermal application of the test substance did not induce an adverse reaction.

Report: KCA 5.2.4/01; [redacted]; 2014; M-484711-01-1
Title: BCS-CN88460 technical - Acute skin irritation study in rabbits
Report No.: 14/069-006N
Document No.: M-484711-01-1
Guideline(s): OECD Test Guideline 404 (2002); EPA OPPTS 870.2500 (1998); EC No 440/2008, B.4 (2008)
Guideline deviation(s): not specified
GLP/GEP: **yes**

Executive Summary

In a study to determine its skin irritation potential, the test item BCS-CN88460 was applied to by topical semi-occlusive application of 0.5g of the test item to the intact shaved flank of 3 male young adult New Zealand White rabbits. The duration of treatment was four hours. The treated skin surface was examined at 1, 24, 48, and 72 hours after patch removal.

There were no observed clinical signs noted on the skin of the treated animals at any time point after removal of the test item. No clinical signs of systemic toxicity were observed in the animals during the study, and no mortalities occurred. The body weights of all rabbits were considered to be within the normal range of variability.

Under the circumstances of this study, BCS-CN88460 was not irritant to the skin as tested in male New Zealand White rabbits. The study does not trigger any classification or labelling under Regulation (EC) No 1272/2008 (CLP), or GHS (rev. 7, 2017).

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% (w/w)
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

2. Vehicle and / or positive control: water for moistening gauze application pad

3. Test animals:	
Species:	Rabbit
Strain:	New Zealand White, male
Age:	Approximately 11 weeks
Weight at dosing:	2701-2764g
Source:	[redacted]
Acclimation period:	5 days
Diet:	Uni diet for rabbits (Agribrands Europe), ad libitum
Water:	Municipal tap water ad libitum
Housing:	Individually in wire rabbit cages
Environmental conditions:	
Temperature:	20 ± 3°C
Humidity:	30-70%
Air changes:	15-20 changes per hour
Photoperiod:	12 hours daily

B. STUDY DESIGN AND METHODS

1. In life dates: 21 February-1 March 2014

2. Animal assignment and treatment

Animals were not assigned to different treatment groups. Initially, a single animal was treated as described below. As no significant irritant effect was observed after the one-hour exposure, the test was completed using the 2 remaining animals with an exposure period of four hours.

Approximately 24 hours prior to compound application, hair was clipped from the back and flanks of the animals with an electric clipper. Any animal with overt signs of skin injury or marked irritation which may have interfered with the interpretation of the results were not used in the test.

On the day of treatment, 0.5g of the test item was placed on a surgical gauze pad and sufficient water was added to dampen the material and ensure good contact with the skin. This gauze pad was applied to the intact skin of the clipped area and was kept in contact with the skin by a patch with a surrounding adhesive, hypoallergenic plaster. The entire trunk of the animals was then wrapped with plastic wrap held in place by an elastic stocking.

After 4 hours, the dressing was removed and the skin was flushed with lukewarm tap water to clean the application site. The skin reaction was assessed at approximately 1, 24, 48, and 72 hours after removal of the dressing, gauze patch, and test item, with the assessment

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scored according to the numerical scoring system listed in Commission Directive 2004/73/EC, based on the Draize scoring system.

Clinical signs were recorded daily. The body weights were recorded on the day of application and at termination of observation.

3. Statistics

Statistical analysis of the results was not required.

II. RESULTS AND DISCUSSION

A. FINDINGS

There were no clinical signs, nor indications of skin irritation effects, at any time point after dermal application of the test substance. The individual values for skin irritation scores are shown in Table 5.2.4/01-1.

Table 5.2.4/01-1: Individual values for skin irritation in three rabbits administered BCS-CN88460 for four hours

Animal	Observation	24h	48h	72h	Mean scores	Response	Reversible (days)
00806	Erythema and eschar	0	0	0	0.00	-	N/A
	Edema	0	0	0	0.00	-	N/A
00811	Erythema and eschar	0	0	0	0.00	-	N/A
	Edema	0	0	0	0.00	-	N/A
00810	Erythema and eschar	0	0	0	0.00	-	N/A
	Edema	0	0	0	0.00	-	N/A

B. DEFICIENCIES

There were no deficiencies in this study.

III. CONCLUSIONS

Under the circumstances of this study, BCS-CN88460 was not irritant to the skin as tested in male New Zealand White rabbits. The study does not trigger any classification or labelling under Regulation (EC) No 1272/2008 (CLP), or GHS (rev. 7, 2017).

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CA 5.2.5 Eye irritation

Report:	KCA 5.2.5/02; [redacted]; 2014; M-488523-01-1
Title:	BCS-CN88460 technical - *In vitro* eye irritation test in isolated chicken eyes
Report No.:	14/069-038CS
Document No.:	M-488523-01-1
Guideline(s):	OECD Guidelines for Testing of Chemicals No. 438 (26th July 2013); EU Commission Regulation (EC) No 1272/2008 (16th December 2008) on CLP; EU Commission Regulation (EC) No 1152/2010 (8th December 2010) amending Regulation (EC) No 440/2008: Method B 48
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Executive Summary

An *in vitro* eye irritation screening study was conducted in isolated chicken eyes using BCS-CN88460. A total of three eyes were used, and an amount of 30 mg of the test substance was applied to the center of the cornea of each eye to cover the entire surface of the cornea. After ten seconds, the cornea and entire surface of the eye was then rinsed with saline. Positive control eyes were treated with 30 mg imidazole, while the negative control eye was treated with 30 µL saline (NaCl, 0.9% w/v).

After rinsing of the eyes, some of the test material remained stuck to the corneal surface; although in the *in vivo* situation blinking might clear the surface of the eye, the movement of the eye lids might also cause abrasion of the corneal surface. Additionally, some fluorescein retention was observed in eyes treated with BCS-CN88460.

Based on the results of this *in vitro* test for eye irritation, the test item cannot be classified as a severe irritant, however it also cannot be classified as a non-irritant. It was concluded that an *in vivo* study was required for classification of the test substance with regard to eye irritation potential.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% w/w
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

2. Vehicle and /or positive control: positive control: imidazole

3. Test animals:	
Species:	chicken
Strain:	ROSS 308
Age:	Unstated
Weight at dosing:	Not relevant
Source:	[redacted]
Acclimation period:	2 hours between collection of heads at slaughter and processing of eyes; 45-60 minutes acclimation prior to treatment
Diet:	Not relevant
Water:	Not relevant
Housing:	Not relevant

Environmental conditions:	
Temperature:	32 ± 1.5°C
Humidity:	Not measured; to maintain humidity, eyes were either kept on wet papers in a closed chamber, or were held under dripping isotonic saline in a closed chamber
Air changes:	Not relevant
Photoperiod:	Not relevant

B. STUDY DESIGN AND METHODS

1. In life dates: 31 March 2014

2. Eye processing, examination, and acclimatization

Chicken heads were collected in a commercial abattoir after slaughter of chickens destined for human consumption. The heads were inspected for appropriate quality, wrapped with saline-moistened tissue paper, and placed in a closed plastic box with 4-5 heads per box. The heads were processed within approximately 2 hours of collection.

For selection of the eyes, the eyelids were carefully cut away with scissors, avoiding damage to the cornea. One small drop of fluorescein solution (2%, w/v) was applied to the surface of the cornea for a few seconds then rinsed off with 20 ml isotonic saline. The fluorescein-treated corneas were then examined with a hand-held slit lamp or slit lamp microscope while the eyes were still in the heads, to ensure that the corneas were not damaged. If the corneas were in good condition, the eyeballs were carefully removed from the orbit by holding the nictitating membrane with a surgical forceps and cutting the eye muscles with bent scissors. Care was taken to not cut the optical nerve too short. The removal process avoided pressure on the eye while removing the eyeball from the orbit, to prevent distortion of the cornea and subsequent corneal effects. Once it was removed from the orbit, each eye was placed onto damp paper and the nictitating membrane was cut away with other connective tissue. The prepared eyes were kept on wet papers in a closed box so that the appropriate humidity was maintained.

For examination and acclimatization, each prepared eye was placed in a steel clamp with the cornea positioned vertically and the eye in the correct relative position as it would have been in the head of the chicken. As the sclera of the chicken eyeball is relatively firm, only slight pressure was needed to fix the eye properly, and excess pressure on the eye by the clamp was avoided. The clamp with the eyeball was transferred to a chamber of the superfusion apparatus and positioned in such a way that the entire cornea was supplied with isotonic saline dripping from a stainless steel tube, at a rate of approximately 3-4 drops per minute. In order to maintain temperature and humidity, the door of the chamber was kept closed except for manipulations and examinations.

The appropriate number of eyes was selected, and after being placed in the superfusion apparatus the selected eyes were examined again by slit lamp microscopy to ensure that they were in good condition. The focus of the slit lamp was adjusted to clearly see the isotonic saline which was flowing on the surface of the cornea. Eyes where either the baseline fluorescein staining score or the corneal opacity score exceeded 0.5 were rejected. The corneal thickness was measured, and any eye deviating by more than 10% from the mean value for all eyes was rejected. In addition, any eyes showing other signs of damage were rejected and replaced. If the selected eyes were appropriate for the test, acclimatization started and lasted approximately 45-60 minutes. During the acclimatization and also the treatment periods, the chambers of the superfusion apparatus were at a controlled temperature of 32 ± 1.5°C.

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3. Base line assessments

At the end of the acclimatization period, a zero-reference measurement was recorded for cornea thickness and opacity to serve as a baseline for each individual eye. It is considered that the corneal thickness should not change by more than -7% to +5% between the start and the end of the acclimatization period. Slight changes of thickness are considered normal when maintaining enucleated eyes; for the eyes used in this study, the changes in corneal thickness ranged from -2.8% to +1.4%. Following the equilibration period, the fluorescein retention was measured. All eyes were considered to be suitable for the assay.

4. Test procedure

After the zero-reference measurements, each eye in its retaining clamp was removed from the chamber, and placed on a layer of tissue with the cornea facing upwards. The eyes were held in horizontal position, while an amount of 30 mg of the test substance was applied to the cornea, attempting to cover the surface of the cornea uniformly with the test substance but taking care not to damage or touch the cornea.

The positive control eyes were treated similarly with 30 mg powdered imidazole, and the negative control eye was treated with 30 µL of physiological saline.

Three test item-treated eyes, three positive control-treated eyes, and one negative control eye were examined during the study.

The time of application was noted, then after an exposure period of 10 seconds from the end of the application the cornea surface was rinsed thoroughly with 20 mL isotonic saline at ambient temperature, taking care not to damage the cornea but attempting to remove residual test item if possible.

5. Observation and evaluation

The control eyes and test eyes were evaluated before the treatment and at approximately 30, 75, 120, 180, and 240 minutes after the post-treatment rinse. Minor variations within approximately ±5 minutes were considered to be acceptable.

Corneal thickness and corneal opacity were measured at all time points. Fluorescein retention was measured on two occasions, at baseline (t=0) and at approximately 30 minutes after the post-treatment rinse. A Haag-Streit Bern 900 slit-lamp microscope was used for the measurements.

Corneal swelling, corneal opacity, and fluorescein retention were determined. At the end of the study, the corneas were carefully removed from the eyes, and preserved in individual containers of 10% neutral buffered formalin for potential histopathology.

II. RESULTS AND DISCUSSION

A. FINDINGS

The test item and the imidazole positive control substance were stuck on the corneal surface after the post-treatment rinse, and surfaces of the corneas on these eyes were not cleared by 240 minutes after the post-treatment rinse.

The mean values of the treated eyes for maximum corneal thickness change, corneal opacity change, and fluorescein retention change are given below, for eyes treated with BCS-CN88460, with imidazole (positive control) or with isotonic saline (negative control). The conclusion on eye irritancy was based on the quantitative assessments specified in the OECD guideline.

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Table 5.2.5/02-1: Observations in isolated chicken eyes treated with either BCS-CN88460, imidazole (positive control), or isotonic saline (negative control)

	BCS-CN88460	Imidazole	Phys. saline
Observation	**Value / ICE Class**	**Value / ICE Class**	**Value / ICE Class**
Mean maximum corneal swelling ≤ 75’	0.0% / I	3.3% / I	0.0% / I
Mean maximum corneal swelling ≤ 240’	0.0% / I	7.1% / II	0.0% / I
Mean maximum corneal opacity	0.00 / I	3.67 / IV	0.00 / I
Mean fluorescein retention	0.50 / I	2.83 / IV	0.00 / I
Other observations	The substance was stuck on all corneal surfaces after the post-treatment rinse. The corneal surface was not cleared by 240 minutes after the post-treatment rinse.		none
Overall ICE Class	3 x I	1x II, 2 x IV	3 x I

B. DEFICIENCIES

No deficiencies were noted in the conduct of this study.

III. CONCLUSIONS

Based on this *in vitro* eye irritation screening study in the isolated chicken eye test with BCS-CN88460, the test item cannot be classified as either a severe irritant or a non-irritant. No conclusion of *in vivo* significance can be made from the adherence of the test item to the cornea, as *in vivo* the eyelids may clear the surface but may also provoke abrasion during blinking. It is concluded that an *in vivo* study is required for classification.

Report: KCA 5.2.5/01; [redacted]; 2014; M-493768-01-1
Title: BCS-CN88460 technical - Acute eye irritation study in rabbits
Report No.: 14/069-005N
Document No.: M-493768-01-1
Guideline(s): OECD Test Guideline 405 (2012)
EPA OCSPP 870.2400 (1998)
EC No 440/2008, B.5 (2008)
JMAFF 2-1-5 (2011)
Guideline deviation(s): not specified
GLP/GEP: **yes**

Executive Summary

An acute eye irritation study was conducted in male New Zealand White rabbits with BCS-CN88460, and the irritant effects were evaluated by the Draize method. An amount of 0.1g of the test item was placed into the conjunctival sac of the left eye of each animal, with the untreated right eye serving as control. The eyes were then examined at 1, 24, 48, and 72 hours after application, with fluorescein staining performed 24 hours before application and 24, 48, and 72 hours after application of the test item.

Conjunctival redness, chemosis, and discharge were noted in all three animals at the 24-hour examination. At 48 hours, all three animals showed a decreased amount of conjunctival redness with no chemosis or discharge noted. All observations had reversed by the 72-hour examination.

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Under the conditions of this study, BCS-CN88460 applied to the mucosa of one eye in each of three rabbits caused conjunctival effects at one hour after application. These effects were fully reversible within 72 hours. The study does not trigger any classification or labelling under Regulation (EC) No 1272/2008 (CLP), or GHS (rev. 7, 2017).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2%, w/w
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

2. Vehicle and / or positive control: none

3. Test animals:	
Species:	Rabbit
Strain:	New Zealand White (male)
Age:	Approximately 15 weeks
Weight at dosing:	3922-3950g
Source:	[redacted]
Acclimation period:	At least 34 days
Diet:	UNI diet for rabbits (Agribrands Europe) ad libitum
Water:	Municipal tap water ad libitum
Housing:	Individually in wire cages
Environmental conditions:	
Temperature:	20 ± 3°C
Humidity:	32-80%
Air changes:	15-20 air changes per hour
Photoperiod:	12 hours daily

B. STUDY DESIGN AND METHODS

1. In life dates: 20-29 May 2014

2. Animal assignment and treatment

There was no specific assignment to treatment groups. Three male rabbits were selected, and any animal with ocular lesions or a positive fluorescein stain prior to installation was rejected.

Initially, only one rabbit was treated with the test item. As the local effect in the first rabbit showed scores of above zero but not severe, then a second rabbit was treated after the 72-hour observation of the first rabbit, and a third rabbit 72 hours after the treatment of the second animal.

Sixty minutes prior to substance application, a systemic opiate analgesic was administered via subcutaneous injection. Five minutes prior to test substance application, a topical ocular anesthetic was applied to each eye, including the control eye to ensure direct comparison of any ocular observations. Eight hours after test substance application, a systemic opiate analgesic and a non-steroidal anti-inflammatory drug (NSAID) were administered via subcutaneous injection. The systemic opiate analgesic was injected at approximately 12

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hours after the post treatment analgesic and the NSAID approximately every 24 hours until the ocular lesions were resolved and no clinical signs of pain or distress were present.
A single dose of 0.1g of the test item was placed in the conjunctival sac of the left eye of each animal, after gently pulling the lower lid away from the eyeball. The lids were then gently held together for at least one second in order to prevent loss of the material.

The eyes were examined at 1, 24, 48, and 72 hours after application. Fluorescein staining (2%) was performed 24 hours before administration of the test item and 24, 48, and 72 hours after treatment to examine corneal damage. The duration of the observation period was sufficient to identify reversibility or irreversibility of any changes noted. All rabbits were examined for distress at least twice daily.
The eye irritation scores were evaluated according to the Draize system.

3. Statistics
No statistical analysis of the results was called for.

II. RESULTS AND DISCUSSION

A. FINDINGS

There were no mortalities, effects on body weight, or clinical signs which could be attributed to ocular administration of BCS-CN88460.
Only slight observations were noted in the treated eyes, as shown in Table 5.2.5/01-1.

Table 5.2.5/01-1: Observations in treated eyes of three rabbits administered BCS-CN88460 in the conjunctival sac of one eye

Animal	Effects	24h	48h	72h	Mean score	Response	Reversible (days)
00978	Corneal opacity	0	0	0	0.00	-	N/A
	Iritis	0	0	0	0.00	-	N/A
	Redness conjunctivae	2	1	0	1.00	-	3
	Chemosis conjunctivae	1	0	0	0.33	-	2
	Discharge	2	0	0	0.67	-	2
00987	Corneal opacity	0	0	0	0.00	-	N/A
	Iritis	0	0	0	0.00	-	N/A
	Redness conjunctivae	2	1	0	1.00	-	3
	Chemosis conjunctivae	1	0	0	0.33	-	2
	Discharge	2	0	0	0.67	-	2
0979	Corneal opacity	0	0	0	0.00	-	N/A
	Iritis	0	0	0	0.00	-	N/A
	Redness conjunctivae	2	1	0	1.00	-	3
	Chemosis conjunctivae	1	0	0	0.33	-	2
	Discharge	2	0	0	0.67	-	2

B. DEFICIENCIES

No deficiencies were noted in the conduct of this study.

III. CONCLUSIONS

Under the conditions of this study, BCS-CN88460 applied to the mucosa of one eye in each of three rabbits caused conjunctival effects at one hour after application. These effects were fully reversible within 72 hours. The study does not trigger any classification or labelling under Regulation (EC) No 1272/2008 (CLP), or GHS (rev. 7, 2017).

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CA 5.2.6 Skin sensitization

Report:	KCA 5.2.6/01; [redacted]; 2015; M-524452-01-1
Title:	BCS-CN88460 technical - Local lymph node assay in the mouse
Report No.:	14/069-037E
Document No.:	M-524452-01-1
Guideline(s):	OECD Guidelines for Testing of Chemicals No. 429, "Skin Sensitisation: Local Lymph Node Assay", Adopted: 22 July 2010; Commission Regulation (EC) No 440/2008 of 30 May 2008, B.42., "Skin Sensitisation: Local Lymph Node Assay" as amended by Commission Regulation (EU) No 640/2012 of 6 July 2012; EPA Health Effects Test Guidelines, OPPTS 870.2600, "Skin Sensitization" (EPA 712-C-03-197, March 2003)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

The aim of this study was to determine the skin sensitization of BCS-CN88460 following dermal exposure, formulated in acetone:olive oil 4:1 (v/v). Five female CBA/J Rj mice per group were allocated to a negative control group which received the vehicle, a positive control group which received 25% α-hexylcinnamaldehyde (HCA), and four groups which received BCS-CN88460 at 5%, 10%, 25%, or 50%.

The formulations of the test item were applied to the experimental animals (25 µL/ear) on study days 1, 2, and 3. On study day 6, the cell proliferation in the local lymph nodes was measured by incorporation of tritiated methyl thymidine (^{3}H-TdR), and the stimulation index was calculated for each group.

No mortality or systemic toxicity was observed during the study. There were no visual signs of local irritancy at the site of application. A minimal amount of test item precipitate was observed on the ears of the animals in the 50% dose group on study days 1-6 and in the 25% dose group on study days 1-3. Alopecia was recorded in the 50% dose group on study days 3-6. No treatment-related effects were observed on body weights in any group.
The observed stimulation index was 1.2, 1.2, 2.5, and 5.6 at concentrations of 5%, 10%, 25%, and 50%, respectively. The calculated EC3 value was 29.0%.

Under the conditions of the study, BCS-CN88460 was shown to have sensitization potential in the mouse Local Lymph Node Assay, with an EC3 value of 29.0%. This triggers classification under Regulation (EC) No 1272/2008 and GHS (rev.7, 2017) as Skin Sens. Category 1B, H317.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2%, w/w
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

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2. **Vehicle and / or positive control:** acetone:olive oil, 4:1 (AOO, v/v)

3. Test animals:	
Species:	Mice (females)
Strain:	CBA/J Rj
Age:	Approximately 12 weeks old
Weight at dosing:	21.8-24.7g
Source:	[redacted]
Acclimation period:	5 days
Diet:	[redacted]® Rat/Mouse Breeding and maintenance diet
Water:	Municipal tap water, ad libitum
Housing:	Individually in polypropylene / polycarbonate cages with Lignocel bedding
Environmental conditions:	
Temperature:	18.6-24.9°C
Humidity:	30-76%
Air changes:	15-20 exchanges per hour
Photoperiod:	12 hours daily

B. STUDY DESIGN AND METHODS

1. **In life dates:** 12 March-12 May 2014

2. **Animal assignment and treatment**
Unique numbers written on the tail of each animal with permanent marker were used to identify the animals; the mice were randomized and assigned to experimental groups. The randomization was checked by computer software according to actual body weights, to verify the homogeneity and variability between groups.

In the preliminary irritation / toxicity test, two animals per dose were exposed to test item concentrations of 100% or 50% in AOO. This preliminary test was terminated on study day 6 without administration of radioactive material. No mortality, signs of systemic toxicity, or marked body weight loss were observed in any of these animals. Test item precipitate was noted on the ears of the animals in both dose groups (in fact at 100% the formulation immediately solidified and the resulting material peeled off from the ears of the animals within several minutes), while minimal alopecia was observed in the animals of the 50% dose group on study days 3-6. There was no visual indication of irritation at the application site. Ear thickness was increased in the 50% dose group. There was no qualitative change in the appearance of the draining auricular lymph nodes. Based on these findings, concentrations of 5%, 10%, 25%, and 50% were used in the definitive study.

In both the preliminary and the definitive study, each mouse was topically dosed with 25 µL of the appropriate formulation of the test item in AOO on the dorsal surface of each ear, using a pipette. Each animal was dosed once a day on study days 1, 2, and 3.

On study day 6 of the definitive study, animals were intravenously injected via the tail vein with sterile phosphate-buffered saline containing approximately 20 µCi of 3H-TdR. Five hours after intravenous injection, the mice were euthanized by asphyxiation with ascending doses of carbon dioxide, followed by cervical dislocation or transection of major cervical blood vessels. The draining auricular lymph nodes were removed and single cell suspensions of lymph node cells were prepared by mechanical disaggregation. The lymph node cells were washed twice in phosphate-buffered saline.

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After the final washing step, the pelleted lymph node cells were gently resuspended and macromolecules were precipitated by the addition of 5% TCA. The suspensions were incubated for approximately 18 hours at 2-8°C, and then centrifuged. The pellets were resuspended in 5% TCA, dispersed, and added to scintillation fluid. Incorporation of 3H-TdR was measured as the number of disintegrations per minute (DPM).

Measured DPM per animal were corrected with the background DPM, which was the average of two measured DPM values of 5% TCA solutions. The results per animal were then expressed as DPN, or DPM divided by the number of lymph nodes. The Stimulation Index, or mean DPN value of a treated group divided by the mean DPN value of the negative control group, was calculated, with SI greater than 3 indicating a positive result.

The test item is regarded if a sensitizer if the SI for at least one dose is greater than 3, and if a conventional dose-response relationship is observed.

3. Statistics

Statistical analysis was performed with the SPSS/PC+ (4.0.1) software. The heterogeneity of variance between groups was checked by Bartlett's test for the measured DPM values. Where no significant heterogeneity was detected, a one-way analysis of variance was carried out. If the result was positive, then Duncan's Multiple Range test was used to assess the significance of inter-group differences. Where significant heterogeneity was found, the normal distribution of examined by Kolmogorow-Smirnow test. In the case of non-normal distribution, the non-parametric method of Kruskal-Wallis One-Way analysis of variance was applied. If a positive result was detected, the inter-group comparisons were performed using the Mann-Whitney U-test.

II. RESULTS AND DISCUSSION

A. FINDINGS

There were no mortalities or systemic toxicity observed during the study, nor was there any visual indication of local irritancy at the site of test item application. Body weight was not affected in any group. A minimal amount of test item precipitate was observed on the ears of the animals in the 50% dose group on study days 1-6 and in the 25% dose group on study days 1-3. Minimal alopecia was recorded in the 50% dose group in study days 3-6.

Ear thickness measurements were increased for some animals at some time points in the treated and the positive control groups. However, biopsy weights were within the historical control range, and it is considered that in some cases test item precipitate remaining on the ear may have interfered with measurements.

Slightly enlarged lymph nodes were recorded (subjective judgement based on observations in previous experiments) for animals in the 50% dose group, and slightly larger than normal lymph nodes were noted in the positive control groups.

The SI exceeded 3 at the top dose of 50%, with a statistically significant increase also observed at 25%. The EC3 value is 29.0%.

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Table 5.2.6/01-1: Mean ear thickness, biopsy weight, DPN, and Stimulation Index in the mouse local lymph node assay

			Treatment group					
Parameter	**Day**	**Ear**	**AOO**	**5%**	**10%**	**25%**	**50%**	**HCA**
Ear thickness, mm	1	Right	0.22	0.21	0.19	0.21	0.21	
		Left	0.21	0.21	0.21	0.22	0.21	0.20
	3	Right	0.22	0.23	0.23	0.23	0.29	0.23
		Left	0.22	0.23	0.22	0.23	0.28	0.24
	6	Right	0.22	0.22	0.21	0.22	0.24	0.24
		Left	0.22	0.22	0.22	0.22	0.24	0.24
Biopsy weight[1], mg			15.44	15.72	14.45	15.62	16.36	16.01
Group DPN			178.3	207.8	206.5	449.8	997.7	2450.8
Stimulation Index			1.0	1.2	1.2	2.5**	5.6**	14.3**

[1] General historical control range: 11.92-22.53mg. Positive response is > 28.16mg.
** statistically significant at $p < 0.01$.

B. DEFICIENCIES

No deficiencies were noted.

III. CONCLUSIONS

Under the conditions of the study, BCS-CN88460 was shown to have sensitization potential in the mouse Local Lymph Node Assay, with an EC3 value of 29.0%. This triggers classification under Regulation (EC) No 1272/2008 and GHS (rev. 7, 2017) as Skin Sens. Category 1B, H317.

CA 5.2.7 Phototoxicity

According to the new data requirements (Commission Regulation (EU) No 283/2013 of 1 March 2013; Official Journal of the European Union, L93/1, 3.4.2013), the conduct of a phototoxicity study and a photomutagenicity study is required under certain conditions.
The circumstances in which a phototoxicity study is required are

Where the active substance absorbs electromagnetic radiation in the range 290-700 nm and is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution. If the ultraviolet / visible molar extinction / absorption coefficient of the active substance is less than 10 L x mol-1 x cm-1, no toxicity testing is required.

The conditions for conduct of a photomutagenicity study are defined in the new data requirements as follows:

Special testing requirements in relation to photomutagenicity may be indicated by the structure of a molecule. If the ultraviolet / visible molar extinction / absorption coefficient of the active substance and its major metabolite is less than 1000 L x mol-1 x cm-1, photomutagenicity testing is not required.

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In Lynch et al (2011)[1], a tiered testing approach for photosafety was considered appropriate, beginning with a phototoxicity study. "If an *in vitro* 3T3 NRU phototoxicity study is negative there is no need for a photogenotoxicity study. Given the similarity of the underlying principles involved in inducing the different endpoints it is very unlikely that a clearly non-phototoxic compound could have a relevant photogenotoxic potential." The recommendation was further made by the Expert Panel that if a positive (phototoxic) response was observed in the *in vitro* 3T3 NRU study, it should be followed up by further *in vitro* or *in vivo* testing for phototoxicity, rather than by testing for photogenotoxicity. In fact, "… the Expert Panel consensus statement was to exclude photogenotoxicity testing as a routine part of the standard photosafety testing strategy."

Based on the approach delineated in Lynch et al. (2011), the clearly negative results of the below-described *in vitro* phototoxicity study, as well as the fact that the ultraviolet / visible molar extinction / absorption coefficient of the active substance is less than 1000 L x mol-1 x cm-1, a photomutagenicity study with isoflucypram is not required and would not bring additional information.

Report:	KCA 5.2.7/01; [redacted] C.; 2018; M-613506-02-1
Title:	Isoflucypram technical: Cytotoxicity assay *in vitro* with BALB/c 3T3 Cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight
Report No.:	1858400
Document No.:	M-613506-02-1
Guideline(s):	OECD 432
Guideline deviation(s):	None
GLP/GEP:	**yes**

As the ultraviolet / visible molar extinction / absorption coefficient of the active substance BCS-CN88460 exceeds the trigger of 10 L x mol-1 x cm-1, a phototoxicity study has been performed *in vitro* using BALB/c 3T3 cells.

Cells were exposed to BCS-CN88460 in either the absence or the presence of UVA filtered light for a period of 50 minutes, then incubated overnight to examine cytotoxicity through use of a Neutral Red medium. The positive control substance chlorpromazine showed the expected phototoxic results. In the definitive study, BCS-CN88460 did not induce cytotoxicity after UVA irradiation, and thus under the conditions of this experiment is considered to be non-phototoxic.

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I. MATERIALS AND METHODS

A. Materials

1. Test material:	
Name:	BCS-CN88460
Synonyms:	Isoflucypram
Description:	beige solid
Lot/Batch no:	2013-006492
Purity:	94.2%, w/w
CAS#:	4255734-28-1
Stability of test compound:	Until 7 June 2018

[1] Lynch et al. (2011): Considerations on photochemical genotoxicity. II: Report of the 2009 International Workshop on Genotoxicity Testing Working Group. Mut. Res., 723(2011) 91-100.

2. Vehicle and positive control:	Solvent control for test item: Earle's Balanced Salt Solution (EBSS) containing 1% (v/v) DMSO Solvent control for positive control: EBSS Positive control: chlorpromazine dissolved in EBSS

3. Test system:		BALB/c 3T3 cell clone c31
	Culture medium:	Dulbecco´s Minimal Essential Medium (DMEM) supplemented with 10% (v/v) newborn calf serum (NCS).
	Cell cultures:	Thawed stock cultures were propagated at 37 ± 1.5 °C in 75 cm^2 plastic flasks. Seeding was done with about 1 x 10^6 cells per flask in 15 mL DMEM, supplemented with 10 % NCS. Cells were sub-cultured twice weekly. The cell cultures were incubated at 37 ± 1.5 °C in a 7.5 ± 0.5% carbon dioxide atmosphere.

B. Study design and methods

1. Treatment

Dose:

Test item	+/– UV	Final concentrations in µg/mL
BCS-CN88460	+/–	0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.50
Positive control*	-	6.25, 12.5, 25, 37.5, 50, 75, 100, 200
	+	0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0
Solvent control	+/–	EBSS containing 1 % (v/v) DMSO

* chlorpromazine

The test item BCS-CN88460 was dissolved in DMSO and added to the culture medium so that the final concentration of DMSO in the medium was 1% (v/v). The concentrations chosen for this study were based on solubility of the test item in the solvent and medium.

Seeding of cultures:

2 x 10^4 cells per well were seeded in 100 µL culture medium in two 96 well plates

Replicates:

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

Treatment & irradiation:

24 h after seeding the cultures were washed with EBSS. 100 µL solved test item added per well for pre-incubation of the plates for 1 hour in the dark. Afterwards one plate was irradiated at 1.65 or 1.8 mW/cm^2 (approximately 5 J/cm^2) for 50 min ± 2 min at 26 °C, the other plate was stored for 50 min ± 2 min at 26 °C in the dark. After irradiation, the test item was removed and both plates were washed twice with EBSS. Fresh culture medium was added and the plates were incubated overnight at 37 ± 1.5 °C and 7.5 ± 0.5 % CO_2.

Light source:

Irradiation was performed with a Dr Hoenle Sol 500 solar simulator, using filter H1 to keep UVB irradiation as low as possible. Wavelength produced with the filter was > 320 nm. UVA intensity was measured throughout the irradiated area with a UV meter, the area of homogeneous irradiation

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was marked, and the marked area was used for irradiation of cultured cells. The distance between light source and marked area was determined as 53 cm. The solar simulator was switched on about 30 minutes prior to the start of the experiment.

Cytotoxicity determination: For measurement of Neutral Red uptake the medium was removed, the cells were washed with EBSS and 0.1 mL serum-free medium containing 50 µg Neutral Red / mL were added to each well. The plates were incubated for another 3 hours at 37 ± 1.5 °C and 7.5 ± 0.5 % CO_2 before the medium was removed completely and the cells were washed with EBSS. For extraction of the dye, 0.15 mL of a solution of 49% (v/v) deionised water, 50% (v/v) ethanol and 1% (v/v) acetic acid were added to each well. After approximately 10 minutes at room temperature and a brief agitation, the plates were transferred to a microplate reader (Versamax® Molecular Devices) equipped with a 540 nm filter to determine the absorbance of the extracted dye. This absorbance showed a linear relationship with the number of surviving cells.

2. Evaluation

The mean absorption (OD_{540}) value per concentration was calculated. The IC_{50}* values were determined by curve fitting by software. The Photo-irritancy factor (PIF), as well as the Mean Phototoxic Effect (MPE) was calculated according to OECD guideline 432.
*IC_{50} = effective dose where only 50% of the cells survived

Evaluation criteria: If PIF < 2 or MPE < 0.1 no phototoxic potential is predicted
If PIF > 2 and < 5 or MPE > 0.1 and < 0.15 a probable phototoxic potential is predicted.
If PIF > 5 or MPE > 0.15 a phototoxic potential is predicted.

II. RESULTS AND DISCUSSION

In the range finding experiment (RFE), a dose-dependent cytotoxicity was observed after treatment of cells with BCS-CN88460 in both the presence and the absence of artificial sunlight. The IC50 of BCS-CN88460 was 27.11 µg/ml with irradiation, and 28.78 µg/ml without irradiation. In the RFE, the PIF of the test item was 1.062 and the MPE was 0.108. This suggested that the test item might have phototoxic properties.
In order to determine whether or not BCS-CN88460 was indeed phototoxic, the same concentrations were used in the main experiment. In the main experiment, the IC50 of BCS-CN88460 was 23.44 µg/ml with irradiation and 22.58 µg/ml without irradiation. The PIF of BCS-CN88460 in this experiment was 0.965, and the MPE was -0.042.

The mean of solvent control values of the irradiated versus the non-irradiated group met the acceptance criteria. The positive control chlorpromazine induced phototoxicity in the expected range in the presence of irradiation.
TheOD_{540} values for each concentration of BCS-CN88460 in the range-finder and main experiment are summarized in Table 5.2.7/01-1 and Table 5.2.7/01-2, respectively.

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Table 5.2.7/01-1: OD_{540} values in the range-finding experiment with BCS-CN88460 and chlorpromazine

Substance	Without UVA			With UVA		
	Concentration, µg/ml	Mean OD_{540nm}	% solvent control	Concentration, µg/ml	Mean OD_{540nm}	% solvent control
Solvent control	-	0.9430*	100.00	-	0.8937*	100.00
BCS-CN88460	0.49	1.0264	108.84	0.49	0.9267	103.69
	0.98	1.0351	109.76	0.98	0.8680	97.13
	1.95	1.0119	107.30	1.95	0.8220	91.98
	3.91	1.0443	110.74	3.91	0.8100	90.64
	7.81	1.0292	109.14	7.81	0.8253	92.35
	15.63	0.9798	103.90	15.63	0.8055	90.14
	31.25	0.3456	36.65	31.25	0.1553	17.38
	62.50	0.1362	14.44	62.50	0.1099	12.30
Chlorpromazine	-	0.9251*	100.00	-	0.7795*	100.00
	6.25	0.9049	97.81	0.125	0.7445	95.52
	12.5	0.7988	86.34	0.25	0.6532	83.80
	25.0	0.1483	16.03	0.50	0.5437	69.75
	37.5	0.0595	6.44	0.75	0.1968	25.25
	50.0	0.0640	6.91	1.00	0.0832	10.67
	75.0	0.0625	6.75	1.50	0.0766	9.83
	100	0.0620	6.70	2.00	0.0763	9.79
	200	0.0601	6.50	4.00	0.0770	9.87

* shows the mean $OD_{540\ nm}$ over 12 wells.

Table 5.2.7/01-2: $OD_{540\ nm}$ values in the main experiment with BCS-CN88460 and chlorpromazine

Substance	Without UVA			With UVA		
	Concentration, µg/ml	Mean OD_{540nm}	% solvent control	Concentration, µg/ml	Mean OD_{540nm}	% solvent control
Solvent control	-	0.9176*	100.00	-	0.8213*	100.00
BCS-CN88460	0.49	0.8817	96.09	0.49	0.8557	104.19
	0.98	0.8889	96.87	0.98	0.8385	102.09
	1.95	0.8748	95.34	1.95	0.8759	106.65
	3.91	0.8723	95.06	3.91	0.8313	101.22
	7.81	0.8357	91.07	7.81	0.8206	99.92
	15.63	0.7984	87.01	15.63	0.7690	93.63
	31.25	0.0713	7.77	31.25	0.0867	10.56
	62.50	0.0663	7.23	62.50	0.0778	9.47
Chlorpromazine	-	0.8633*	100.00	-	0.8557*	100.00
	6.25	0.8177	94.72	0.125	0.8227	96.14
	12.5	0.3205	37.12	0.25	0.7170	83.78
	25.0	0.0923	10.69	0.50	0.3946	46.12
	37.5	0.0707	8.18	0.75	0.0883	10.32
	50.0	0.0782	9.06	1.00	0.0663	7.75
	75.0	0.0758	8.77	1.50	0.0744	8.69
	100	0.0763	8.84	2.00	0.0754	8.81
	200	0.0748	8.67	4.00	0.0774	9.05

* shows the mean $OD_{540\ nm}$ over 12 wells.

The IC_{50}, PIF and MPE values for the range-finding experiment and the main experiment are summarized in Table 5.2.7/01-3 below.

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Table 5.2.7/01-3: Summary of results of the Neutral Red assay in the range-finding and main experiments conducted with BCS-CN88460

	Substance	IC_{50} (+ UV) µg/ml	IC_{50} (- UV) µg/ml	PIF	MPE
Range-finding experiment	BCS-CN88460	27.11	28.78	1.062	0.108
	Chlorpromazine	0.596	18.18	30.480	0.568
Main experiment	BCS-CN88460	23.44	22.58	0.965	- 0.042
	Chlorpromazine	0.4592	11.74	25.576	0.647

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

III. CONCLUSIONS

In conclusion, in this study and under the experimental conditions reported, the test item BCS-CN88460 does not possess any phototoxic potential.

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CA 5.3 Short-term toxicity

Table 5.3-1: Summary of Isoflucypram short term toxicity studies

Study	NOAEL	LOAEL	Effects
Rat 28-day study Diet incorporation 0, 300, 1000, 3000 ppm [redacted]; 2017; M-464024-03-1	300 ppm 22.8 / 25.6 mg/kg bw/d males / females	1000 ppm 83.3 / 86.5 mg/kg bw/d males / females	**Liver:** increased absolute, relative wt, panlobular hypertrophy, activation of CAR, PXR, induction of specific hepatic enzymes. **Thyroid:** increased absolute, relative wt, follicular cell hypertrophy **Kidney:** male-rat-specific α_{2u}-globulin accumulation, tubular hyaline droplets
Mouse 28-day study Diet incorporation 0, 200, 800, 2000 ppm [redacted]; 2012; M-442490-01-1	Females: NOAEL = 800 ppm (149 mg/kg bw/day) Males: NOEL = 200 ppm (32 mg/kg bw/day)	Females: 2000 ppm (374 mg/kg bw/day) Males: 800 ppm (133 mg/kg bw/day)	Increased liver weight, increased activities of ASAT, ALAT, AP; increased incidene of hepatocellular hypertrophy, hepatocellular necrotic foci, hepatocellular single cell necrosis
Dog 28-day study Diet incorporation 0, 300, 1000, 3000 ppm [redacted]; 2014; M-503716-01-1	Females: NOAEL = 1000 ppm (36.5 mg/kg bw/day) Males: NOEL = 1000 ppm (37.7 mg/kg bw/day)	3000 ppm 76.9 / 90.2 mg/kg bw/d males / females	Decreased body wt / body wt gain; Increased liver wt, hepatocellular hypertrophy, decreased hepatocellular glycogen accumulation
Rat 90-day study Diet incorporation 0, 100, 300, 1000 ppm [redacted]; 2017; M-487478-02-1	300 ppm 18.4 / 21.9 mg/kg bw/d males / females	1000 ppm 63.5 / 80.9 mg/kg bw/d males / females	**Liver:** Increased wt, increased cholesterol in females, enlarged liver, hepatocellular hypertrophy in females only **Urinary tract (males only):** Cellular casts in urine, basophilic tubules, hyaline droplets, male-rat-specific α_{2u}-globulin accumulation, tubular hyaline droplets

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Study	NOAEL	LOAEL	Effects
Mouse 90-day study Diet incorporation 0, 100, 300, 1000 ppm [redacted]; 2013; M-472773-01-1	300 ppm 51.0 / 59.8 mg/kg bw/d males / females	1000 ppm 168 / 207 mg/kg bw/d males / females	Increased liver wt, change from diffuse to mainly centrilobular hepatocellular vacuolation
Dog 90-day study Diet incorporation 0, 170, 500, 1500 ppm [redacted]; 2015; M-520001-01-1	500 ppm 15.9 / 16.2 mg/kg bw/d males / females	1500 ppm 50.4 / 54.0 mg/kg bw/d males / females	Decreased body wt gain, increased alkaline phosphatase, increased liver wt, increased hepatocellular hypertrophy (males only)
Dog 1-year study Diet incorporation 0, 150, 600, 1800 ppm [redacted]; 2017; M-601188-01-1	150 ppm 4.2 mg/kg bw/day in both sexes	600 ppm 18.8 / 17.6 mg/kg bw/d males / females	Decreased body wt, body wt gain; increased alkaline phosphatase, increased liver wt, increased hepatocellular hypertrophy

In short-term studies, the liver was identified as the principal target organ in all species. Additional target organs included the thyroid in the rat and the kidney in the male rat.

In the rat 28-day study, dietary administration of BCS-CN88460 at concentration of 0, 300, 1000, and 3000 ppm, providing doses of 0, 22.8, 83.3, and 240 mg/kg bw/day in males and 0, 25.6, 86.5, and 285 mg/kg bw/day in females, did not provoke any mortalities or clinical signs in either sex. Slight decreases were noted in total leucocyte count and in lymphocytes in males at 3000 ppm, as well as a slight increase in erythrocyte count as well. However, as these changes were not observed in other studies, they can be considered to be toxicologically not relevant. Total cholesterol, total protein, and albumin concentrations were increased in males at 3000 ppm. Total bilirubin was decreased in males at 1000 and 3000 ppm and in females at all doses; this is not a toxic effect, but can be considered an indicator of administration of a compound such as BCS-CN88460. Body weight and body weight gain were significantly reduced at 3000 ppm in males only, and terminal body weight was decreased in males at 3000 ppm. Absolute and / or relative liver weights were increased in both males and females from 1000 ppm, accompanied by an increased incidence of enlarged or dark livers and in females prominent lobulation of the liver. The incidence of hepatocellular hypertrophy was increased in both sexes, with a slightly greater effect in females than in males. Relative thyroid weight was increased in males, and in both males and females there was an increase in the incidence of thyroid follicular cell hypertrophy. Measurement of hepatic cytochrome P450 and UDPGT enzyme activity in livers of male and female rats demonstrated, with marked induction of PROD and BROD activities in males and BROD in females, as well as significant induction of T4-UDPGT in both sexes, that BCS-CN88460 acts in the liver and secondarily in the thyroid as a CAR-PXR-activating compound. Finally, specific staining of kidney sections from male rats showed a dose-related increase from the low dose of 300 ppm in the accumulation of alpha2u-globulin droplets in the renal proximal tubules, correlated with an increase in the increased incidence and severity of hyaline droplet accumulation in the proximal tubules. The NOAEL was considered to be 300 ppm (22.8 mg/kg bw/day in males and 25.6 mg/kg bw/day in females) in this 28-day dietary study in the rat.

In the rat 90-day dietary study, the doses were set on the basis of the liver, thyroid, and kidney findings observed in the 28-day rat study. There, liver and thyroid were demonstrated to be target organs in both males and females, with the primary liver effect of increased weight, centrilobular hypertrophy, and CAR-PXR-mediated induction of cytochrome P450 and UDPGT leading to the

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secondary thyroid effect of increased weight and / or increased incidence of thyroid follicular cell hypertrophy. The kidney was also a target in male rats, with an accumulation of alpha2u-globulin and hyaline droplet accumulation in the proximal tubules at high doses. Thus, dietary concentrations of 0, 100, 300, and 1000 ppm were chosen for this dietary study in order to elicit sufficient but not excessive toxicity in the liver, thyroid, and potentially kidney. These concentrations provided systemic doses of 0, 6.34, 18.4, and 63.5 mg/kg bw/day in males and 0, 7.92, 21.9, and 80.9 mg/kg bw/day in females. There was no effect on clinical signs, mortalities, body weight or body weight gain, food consumption, or hematological parameters in either males or females. Total bilirubin was decreased in both males and females, with a greater effect in females, while total cholesterol was slightly increased only in females at 1000 ppm. Urinalysis showed cellular casts in urine of male rats at 1000 ppm. At 1000 ppm, terminal body weight was slightly decreased in both sexes, absolute and relative liver weights were increased in both sexes, and relative thyroid weight was increased in males only. The increased liver weight was accompanied in both sexes by enlarged livers. In females only, at 1000 ppm there was an increase in the incidence of hepatocellular hypertrophy. In both males and females there was an increase in the incidence of thyroid follicular cell hypertrophy and colloid alteration. In males, the incidence and severity of hyaline droplets, basophilic tubules, and granular casts in the kidney was increased at 1000 ppm, and was accompanied by increased accumulation of alpha2u-globulin in the proximal tubules. The NOAEL for this study was 300 ppm (18.4 mg/kg bw/day in males, 21.9 mg/kg bw/day in females).

In the mouse 28-day study, dietary administration of BCS-CN88460 at concentrations of 0, 200, 800, and 2000 ppm (0, 32, 133, and 330 mg/kg bw/day in males and 41, 149, and 374 mg/kg bw/day in females) did not cause any mortalities or clinical signs during the study. Total bilirubin was decreased in all groups in females, although this is an indicator of exposure rather than a toxic effect. The activities of ASAT and ALAT were increased in both sexes, and alkaline phosphatase was increased in males but not in females. There was no biologically significant effect on body weight, body weight gain, or terminal body weight. Absolute and relative liver weights were increased in both males and females at 2000 ppm, accompanied in females by dark liver noted at necropsy, and by an increase in both sexes in the incidence of hepatocellular hypertrophy, single cell necrosis, and necrotic focus(i). In males, hepatocellular single cell necrosis and necrotic focus(i) was also increased at 800 ppm. The NOAEL was considered to be 800 ppm (149 mg/kg bw/day) in females and 200 ppm (32 mg/kg bw/day) in males.

For the mouse 90-day dietary study, the dietary concentrations to be used were set on the basis of the findings in liver observed in male and female mice in the 28-day dietary study. In that study, there were slight effects on the liver of males at 800 ppm (increased ASAT activity, increased necrotic focus(i) and focal single cell necrosis) and in both males and females at 2000 ppm (increased liver weight, increased ASAT, ALAT, and alkaline phosphatase activities, and increased incidence of histopathological findings. Based on those observations, the dietary concentrations for this 90-day dietary study were 0, 100, 300, and 1000 ppm, providing systemic doses of 0, 17.0, 51.0, and 168 mg/kg bw/day in males and 0, 19.5, 59.8, and 207 mg/kg bw/day in females. There was no effect on body weight, body weight gain, food consumption, or terminal body weight in either males or females. Total bilirubin was slightly decreased at 1000 ppm in both males and females, and albumin and total cholesterol were decreased only in males at 1000 ppm. Absolute and / or relative liver weight was increased in males from 300 ppm and in females at 1000 ppm only. The incidence of mainly centrilobular hepatocellular vacuolation was increased in males at 1000 ppm only and in females from 300 ppm, while the incidence of diffuse hepatocellular vacuolation was conversely decreased in both sexes. The NOAEL for this study was set at 300 ppm (51.0 mg/kg bw/day in males, 59.8 mg/kg bw/day in females).

In the dog 28-day dietary study, administration of BCS-CN88460 at concentrations of 0, 300, 1000, and 3000 ppm (providing doses of 0, 12.7, 37.7, and 76.9 mg/kg bw/day in males and 0, 11.3, 36.5, and 90.2 mg/kg bw/day in females) led to decreased food consumption at 3000 ppm in both males and females, accompanied by body weight loss in most animals in that group. Alkaline phosphatase was increased in most animals at 3000 ppm, and total cholesterol was decreased in one male and one female at 3000 ppm. At 3000 ppm, terminal body weight was decreased in females, and absolute and

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relative liver weights were increased in both sexes. This was accompanied by the macroscopic finding of enlarged liver in one male, and by hepatocellular hypertrophy in both males and females, as well as accumulation of brown pigment in Kupffer cells in females only. There were no effects observed in other organs in either sex. The NOAEL was thus established at 1000 ppm, or 37.7 mg/kg bw/day in males and 36.5 mg/kg bw/day in females.

In the dog 90-day dietary study, the dietary concentrations of BCS-CN88460 to be used were established on the basis of the decreased body weight or body weight loss, and increased liver weight and histopathology including hepatocellular hypertrophy and decreased glycogen accumulation. The dietary concentrations for the 90-day study were therefore set at 0, 170, 500, and 1500 ppm, providing systemic doses of 0, 5.5, 15.9, and 50.4 mg/kg bw/day for males and 0, 5.5, 16.2, and 54.0 mg/kg bw/day for females. At the end of the study, at 1500 ppm body weight was slightly reduced while body weight gain was reduced in a toxicologically significant manner in both sexes, although there was no effect on food consumption at any dose in either sex. Alkaline phosphatase was markedly increased in both males and females at 1500 ppm, and bilirubin was slightly decreased in both sexes from 500 ppm (males) or from 170 ppm (females), confirming that the liver was the primary target organ of BCS-CN88460 in the dog. At 1500 ppm, absolute and relative liver weights were increased in both sexes, although this increase was only statistically significant in males, in which it was also accompanied by an increase in the incidence of hepatocellular hypertrophy. The NOAEL for this study was set at 500 ppm (15.9 mg/kg bw/day in males, 16.2 mg/kg bw/day in females).

For the one-year dog study, the dietary concentrations of BCS-CN88460 to be used were established on the basis of the observation at 1500 ppm in the 90-day study of decreased body weight gain, the increase in both sexes of alkaline phosphatase activity, and increase in males of liver weight and centrilobular hepatocellular hypertrophy. The dietary concentrations chosen for the one-year study were therefore 0, 150, 600, and 1800 ppm, providing systemic doses of 0, 4.2, 18.8, and 60.2 mg/kg bw/day in males and 0, 4.2, 17.6, and 49.8 mg/kg bw/day in females. At 1800 ppm, body weight and body weight gain were occasionally reduced, and in females there was a decrease in food consumption. At 1800 ppm, alkaline phosphatase was increased in both males and females. Total bilirubin was decreased in both sexes, in males from 600 ppm and in females from 150 ppm. Analysis of concentrations of BCS-CN88460 and its key metabolites BCS-CX99798 and BCS-CX99799 showed that the compound was well-absorbed and was metabolized in both sexes. There was no toxicologically significant effect on terminal body weight. At 1800 ppm in males, absolute and relative liver weight were increased, while enlarged liver was noted in the majority of males and in one female. There was an increase in the incidence of hepatocellular hypertrophy from 600 ppm in both sexes, and in focal Kupffer cell pigmentation at 1800 ppm only. The NOAEL for the one-year dog study was set at 150 ppm (4.2 mg/kg bw/day in both sexes).

Toxicokinetic measurements in short-term rodent studies

In the 28-day and 90-day studies conducted in the rat and mouse with BCS-CN88460, bioanalytic measurements were not taken and toxicokinetic analysis was not conducted. The in-life phases of these studies preceded the publication date of the relevant guideline (Commission Regulation (EU) No. 283/2013 of 1 March 2013) setting out data requirements for active substances.

The data obtained from the rat developmental toxicity study and the 90-day timepoint of the rat chronic study can be used to give a general idea of the behavior of the compound in the rodent after oral administration. Although toxicokinetic measurements were made in the mouse chronic study, at the 90-day timepoints the metabolites of interest had not yet been identified and thus only the concentration of BCS-CN88460 was measured at that timepoint. Because there is more data available in the rat than in the mouse, the data developed in the rat will be used as an indicator of compound behavior in the mouse as well. Drawbacks to this general approach include the difference between routes of administration (oral gavage for the developmental toxicity compared to dietary incorporation for the 28-day studies in the rat and mouse), the difference in treatment duration (14 days in the rat developmental toxicity study versus 28-day studies in the rat and mouse), and the comparison across species from the rat developmental toxicity study to the 28-day mouse study.

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None the less, the available data can be used as a general guide. As can be seen in Tables 5.3-2 (males) and 5.3-3 (females), the concentration of BCS-CN88460 in plasma generally increases with increasing dose, although there is variation between studies. Similarly, the concentrations of the two metabolites generally increase with an increasing dose of the parent compound in both males and females. Although measurement of toxicokinetic parameters in the 28-day and 90-day studies would have made the studies more directly interpretable, the information obtained in other studies in the rat can be used to aid in the interpretation of the 28-day and 90-day studies.

Table 5.3-2: Systemic dose in mg/kg bw/day and study duration from studies conducted in male rats, and toxicokinetic data from the 90-day time point of the chronic study and from the F0 and F1 adults of the 2-generation reproduction study

		Analyte		
Study	**mg/kg bw/day**	**BCS-CN88460**	**BCS-CX99799**	**BCS-CX99798**
90-day / chronic[1]	1.97	< LOQ	0.068	0.029
90-day	6.3			
90-day / chronic	9.9	< 0.010	0.282	0.162
2-generation F0	11.3	0.0046	0.095	0.138
2-generation F1	13.9	0.0048	0.144	0.174
90-day	18.4			
28-day	22.8			
90-day / chronic	29.3	< 0.019	0.526	0.510
2-generation F0	34.1	0.0089	0.281	0.509
2-generation F1	41.6	0.0082	0.353	0.451
90-day	63.5			
28-day	85.3			
2-generation F0	94.4	0.0238	0.557	1.343
2-generation F1	108.6	0.0210	0.895	1.547
28-day	240			

[1] 90=day / chronic = 90-day timepoint of the chronic study

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Table 5.3-3: Systemic dose in mg/kg bw/day and study duration from studies conducted in female rats, and toxicokinetic data from the 90-day time point of the chronic study, the developmental toxicity study, and the F0 and F1 adults of the 2-generation reproduction study

		Analyte		
Study	**mg/kg bw/day**	**BCS-CN88460**	**BCS-CX99799**	**BCS-CX99798**
90-day / chronic[1]	2.5	< LOQ	0.132	0.014
90-day	7.9			
90-day / chronic	12.4	< 0.012	0.339	0.146
2-generation F0	13.0	0.0153	0.086	0.094
2-generation F1	13.9	0.0274	0.280	0.166
90-day	21.9			
Developmental toxicity	25	< 0.012	0.339	0.039
28-day	25.6			
2-generation F0	40.8	0.0194	0.358	0.541
2-generation F1	41.6	0.0315	0.596	0.455
90-day / chronic	66.2	0.015	0.610	1.113
90-day	80.9			
28-day	86.5			
2-generation F0	104.4	0.0243	1.291	2.073
2-generation F1	108.6	0.0324	1.660	1.583
Developmental toxicity	125	0.015	0.273	0.147
28-day	285			
Developmental toxicity	625	0.017	0.607	0.676

[1] 90=day / chronic = 90-day timepoint of the chronic study

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CA 5.3 Short-term toxicity

CA 5.3.1 Oral 28-day study

Report:	KCA 5.3.1/01; [redacted]; 2017; M-464024-03-1
Title:	BCS-CN88460 - Exploratory 28-day toxicity study in the rat by dietary administration
Report No.:	M-464024-03-1
Document No.:	M-464024-03-1
Guideline(s):	none
Guideline deviation(s):	none
GLP/GEP:	**no**

Executive Summary

BCS-CN88460 was administered continuously via the diet to groups of Wistar rats (five per sex per group) for at least 28 days at concentrations of 0, 300, 1000, and 3000 ppm (equating to 0, 22.8, 83.3, and 240 mg/kg bw/day in males and 0, 25.6, 86.5, and 285 mg/kg bw/day in females, respectively). All animals were observed for mortality and clinical signs daily, body weight and food consumption were measured on a weekly basis, and a detailed physical examination was performed each week during the study. Before necropsy, a blood sample was collected from the retro-orbital venous plexus of each surviving animal for hematology and clinical chemistry determinations. All animals were necropsied, selected organs were weighed, and a range of tissues were taken, fixed, and examined microscopically. The remaining portions of the liver were homogenized for microsomal preparation in order to determine Phase I and Phase II enzyme activities. In addition, intra-abdominal fat from all animals was collected and frozen at approximately -20°C for subsequent analysis in order to evaluate the potential for BCS-CN88460 to accumulate in this tissue.

Dietary administration of BCS-CN88460 for at least 28 days to male and female Wistar rats at dietary concentrations of 300, 1000, and 3000 ppm did not cause any mortalities or treatment-related clinical signs, and no changes in food consumption were observed.

At 3000 ppm (equating to 240 and 285 mg/kg bw/day in males and females, respectively)
Mean body weight was reduced in males by 10-15% throughout the study, when compared to control values. Mean body weight gain per day was reduced in study weeks 1, 2, and 3 by 66%, 18%, and 56% respectively when compared to the controls. The overall mean cumulative body weight gain was reduced by 31%. In females, mean body weight parameters were not affected by dietary administration of BCS-CN88460.

Hematology assessment showed a tendency towards lower total leucoctye counts, lower absolute lymphocyte counts, and slightly higher mean erythrocyte counts in males compared to controls. Clinical chemistry evaluation revealed higher mean total cholesterol, total protein, and albumin concentrations in males compared to controls. Lower mean total bilirubin concentrations were also observed in both sexes copared to controls.

At necropsy, mean terminal body weight was lower in males compared to controls. Mean absolute and relative liver weights were 13-31% higher in males and 61-64% higher in females when compared to controls. Mean absolute and relative thyroid gland weights were 21-41-% higher in males and 20-22% higher in females when compared to controls. Macroscopic observation showed enlarged and / or dark liver in some animals of both sexes, together with prominent lobulation in some females. At the microscopic examination, treatment-related findings were noted in the liver, kidney, and thyroid gland. In the liver, panlobular hypertrophy and hepatocellular periportal microvacuolations were seen in both sexes. In the thyroid gland, minimal to slight follicular cell hypertrophy was noted in both sexes. In the kidney, tubular hyaline droplets were noted in all males together with an increased incidence of bilateral basophilic tubules.

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Analysis of intra-abdominal fat showed that BCS-CN88460 has no potential to accumulate in fat up to the highest dietary concentration, as only traces of BCS-CN8460, below the limit of validation of 50 µg/L, were detected in 4/5 males and 4/5 females.

Hepatic enzyme induction measurements in males showed high induction of BROD and PROD Phase I activities, as well as bilirubin- and 4-nitrophenol-UDPGT Phase II activities. Slight increases were also noted for total cytochrome P450, EROD, and T4-UDPGT activities. In females, the greatest increases were noted for BROD activity and bilirubin-, 4-nitrophenol-, and T4-UDPGT activities, while PROD activity was slightly increased.

At 1000 ppm (equating to 86.5 and 83.3 mg/kg bw/day in male and females, respectively)
Clinical chemistry showed decreased mean total bilirubin concentrations in both males and females compared to controls.

At necropsy, mean absolute and relative liver weights were 12-16% higher in males and 27-30% higher in females when compared to controls. Macroscopic observation revealed enlarged liver in a few animals of both sexes, together with dark liver in some females. At the microscopic examination, panlobular hypertrophy was noted in the liver of one female only, whilst tubular hyaline droplets were noted in the kidney of all males together with an increased incidence of bilateral basophilic tubules.

Measurement of hepatic enzyme activity in males showed the greatest increases in BROD and bilirubin- and 4-nitrophenol-UDPGT activities. Slight increases were also noted in total cytochrome P450 and in EROD, PROD, and T4-UDPGT activities when compared to controls. In females, BROD and bilirubin- and T4-UDPGT activities showed the greatest increases, while PROD activity was slightly increased.

At 300 pm (equating to 22.8 and 25.6 mg/kg bw/day in males and females, respectively)
Clinical chemistry showed lower mean total bilirubin concentrations in both sexes (-19% in males and -44% in females) compared to controls. However, decreased total bilirubin concentrations are considered not to be adverse effects of the test item as they do not represent any functional impairment in the animal.

At necropsy, microscopic examination revealed tubular hyaline droplets in the kidney of 3/5 males. As this change was observed in isolation rather than in association with bilateral basophilic tubules, it was considered not to be adverse.

Hepatic enzyme activity was not induced in males, while a slight increase in bilirubin-UDPGT activity was noted in females, although this induction was considered not to be adverse due to the low magnitude of the change.

In conclusion, the dose level of 300 ppm (equating to 22.8 mg/kg bw/day in males and 25.6 mg/kg bw/day in females) administered to the Wistar rat by the dietary route for at least 28 days was considered to be a No Observed Adverse Effect Level (NOAEL) in both sexes.

BCS-CN88460 was found to be a potent inducer of cytochrome P450 2B (in males only) and 3A, and of 4-nitrophenol-, bilirubin-, and T4-UDPGT activities.

Hyaline droplet nephropathy is a recognized lesion of the male kidney that is related to the accumulation of alpha2u-globulin in the proximal tubules of affected animals. This protein is naturally reabsorbed and acucmulated in the renal tubular epithelium of the young male rat. The sequance of renal events leading to its increased accumulation is dependent upon the interaction between a chemical subtance and alpha2u-globulin and is specific to the male rat. As humans secrete alpha2u-globulin in only trace amounts, this mechanism is generally accepted as being not relevant to

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humans. Therefore, although the effects observed here in the kidney of male rats were considered to be treatment-related, they are considered not to be toxicologically relevant to humans.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	White solid
Lot / Batch #:	NLL 8674-19-4
Purity:	98.6%
CAS #	1255734-28-1
Stability of test compound:	Until 15 June 2012

2. Vehicle and / or positive control: none (diet incorporation)

3. Test animals:	
Species:	Rat
Strain:	Wistar Rj.WI
Age:	Approximately 7 weeks at start of treatment
Weight at dosing:	243-263g (males), 168-189g (females)
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	A04CP1-10 powdered and irradiated diet, [redacted], [redacted], France, ad libitum
Water:	Municipal tap water, ad libitum
Housing:	Individually in polycarbonate and wire-mesh cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 air changes per hour
Photoperiod:	12 hours

B. STUDY DESIGN

1. In life dates: 10 May-15 June, 2012

2. Animal assignment and treatment

On the day of randomization, all animals were weighed. A computerized randomization procedure (Pristima, version 6.3.2 build 17, Xybion Corp.) was used to select animals for the study from the middle of the weight range of the available animals, which ensured a similar body weight distribution among groups for each sex. Animals were assigned permanent identification numbers within groups following randomization.

Diets containing BCS-CN88460 at concentrations of 0, 300, 1000, or 3000 ppm were provided ad libitum to the animals for at least 28 days. These dose levels were set after evaluation of a 14-day toxicity study with BCS-CN88460, where the compound was administered continuously via the diet to groups of 5 male and 5 female Wistar rats at concentrations of 0, 2400, 4800, and 8400 ppm. There was a dose-related decrease, of marked severity from 4200 ppm, in mean body weight gain and food consumption, and absolute and relative liver weights were markedly increased from 4200 ppm. At 2400 ppm, body weight gain and food consumption were only slightly affected, while absolute and relatively liver weights were moderately to strongly increased.

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3. **Diet preparation and analysis**
The test item was incorporated into the diet by dry mixing to provide the required dietary concentrations. When not in use, the diet formulations were stored at room temperature. Diet was prepared once at each concentration for the duration of the study.

The stability of the test item in the diet was demonstrated in a previous study at concentrations of 60 and 10000 ppm for 100 days at room temperature, or 90 days frozen followed by 10 days at room temperature. The homogeneity of BCS-CN88460 in diet was verified for the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as the measured concentration. Dietary levels of the test item were verified for each concentration.

4. **Statistics**
Mean and standard deviation were calculated for each parameter and each group. All statistical analyses were carried out separately for males and females, and group means were compared at least at the 5% level of significance. Statistical analyses were carried out using Pristima, version 6.3.2 build 17, Xybion Corp. as follows for:

- Body weight change parameters;
- Terminal body weight, absolute and relative organ weights;
- Hematology and clinical chemistry parameters; and
- Cytochrome P450 content and liver enzyme activities

Non-significant Bartlett tests were followed by ANOVA; if this was negative, no further statistical analysis was conducted. If ANOVA was positive, it was followed by a two-sided Dunnett test. Significant Bartlett tests were followed by Kruskal-Wallis tests; if this was negative, no further statistical analysis was conducted. If Kruskal-Wallis test was positive, it was followed by a two-sided Dunn test.

For body weight and food consumption parameters, and for liver enzyme activities, if the initial Bartlett test was positive, data were transformed using the log transformation, while the square root transformation was used for hematology parameters. This was followed by a Bartlett test on the transformed data; positive and negative results were then handled as described above.

C. METHODS

1. **Observations**
All animals were checked for moribundity and mortality twice daily on weekdays, and once daily on weekends or public holidays, and at least once daily for clinical signs. 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.

2. **Body weight**
Each animal was weighed at least weekly during the acclimatization period, on the first day of test item administration, then at weekly intervals throughout the treatment period. Additionally, diet-fasted animals were weighed before scheduled necropsy for terminal body weight.

3. **Food consumption and compound intake**
The weight of food supplied and that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any food spillage was noted. The weekly mean achieved dosage intake in mg/kg bw/day for each week and for weeks 1 to 4 was calculated for each sex.

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4. Ophthalmoscopic examination

Ophthalmoscopic examination was not conducted in this study.

5. Hematology and clinical chemistry

On study day 29 or 30, diet was removed from the cages and animals were diet-fasted overnight prior to blood sampling on study day 30 or 31. Animals were anesthetized by isoflurane inhalation and blood samples were collected by puncture of the retro-orbital venous plexus. Standard hematology and clinical chemistry parameters were measured and blood smears were prepared for possible examination if the hematology parameters were abnormal.

6. Urinalysis

Urinalysis was not conducted.

7. Sacrifice and pathology

Animals were diet-fasted prior to sacrifice on study day 30 or 31 by exsanguination under deep isoflurane inhalation anesthesia. All animals were necropsied, including examination of all major organs, tissues, and body cavities. Macroscopic abnormalities were recorded, sampled, and examined microscopically. The adrenal gland, brain, kidney, liver, ovary, pituitary gland, spleen, thyroid gland with parathyroid gland, testis, and uterus including cervix were weighed, with paired organs weighed together.

The adrenal gland, epididymis, kidney, liver, ovary, pituitary gland, spleen, testis, thyroid gland with parathyroid gland, uterus including cervix, and vagina, as well as any macroscopic abnormalities, were fixed in 10% formalin (testis and epididymis were fixed in Davidson's fixative) prior to preparation of histological sections for all animals in all groups. All tissues listed here were examined for animals in the control and high dose groups, while kidney, liver, thyroid gland, vagina, uterus, and macroscopic findings were examined in the intermediate dose groups.

In addition, kidney sections were also stained for α-2-μglobulin in males from the control and high dose groups. Paraffin sections were pre-treated with heat and protease, and monoclonal rat antibodies against α-2-μglobulin were used. The immunohistochemical reaction was followed by incubation with a secondary biotinylated antibody, ultra-detection with streptavidin-horseradish peroxidase complex, and visualization with the chromogen diamino-benzidine.

Intra-abdominal fat from all males and females was collected and frozen at approximately -20°C for subsequent analysis, in order to evaluate the potential for BCS-CN88460 to accumulate in the fat.

8. Hepatotoxicity testing

At final necropsy, portions of liver from all animals were homogenized for microsomal preparations in order to determine the hepatotoxic potential of the test item.

Total cytochrome P450 content in the microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. A single quantification was performed for each sample. Specific cytochrome P450 activities were evaluated by spectrofluorimetry using ethoxyresorufin (EROD, cytochrome P450 1A), pentoxyresorufin (PROD, cytochrome P450 2), and benzoxyresorufin (BROD, cytochrome P450 3A). Phase II enzymatic activities were determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol and bilirubin as substrates. Thyroxine (T4) glucuronidation

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activity was determined through incubation of 125I-thyroxine with liver microsomes and determination of peak areas of both T4 and T4-glucuronide.

II. RESULTS AND DISCUSSION

A. CLINICAL SIGNS AND MORTALITY

1. Clinical signs of toxicity

No treatment-related clinical signs were observed during the study at any dose level.

2. Mortality

There were no mortalities observed during the study.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 3000 ppm in males, mean body weight was statistically significantly reduced throughout the study, with overall mean cumulative body weight gain also significantly reduced; clearly, 3000 ppm caused excessive toxicity in males. In females, mean body weight and body weight gain were not affected by dietary administration of BCS-CN88460. There were no treatment-related effects on body weight or body weight gain at either 1000 or 300 ppm. Body weight and body weight gain data are shown in Table 5.3.1/01-1.

Table 5.3.1/01-1: Mean body weight and body weight gain in male and female rats administered BCS-CN88460 via the diet for four weeks

		BCS-CN88460, dietary concentration in ppm							
		Males				Females			
	Day	0	300	1000	3000	0	300	1000	3000
Body wt, g	0	254.4	254.6	255.1	254.8	175.6	176.8	174.9	177.4
	8	301.7	302.9	293.5	271.1**	185.7	187.7	189.8	187.7
	15	348.0	356.6	346.0	309.0**	195.9	205.8	207.3	207.8
	22	378.0	388.8	378.4	323.3**	209.2	210.5	215.6	206.1
	29	402.7	418.4	402.1	356.8**	223.4	223.1	224.6	226.5
Body wt gain, g	1-29	148.4	163.8	147.0	102.1**	47.8	46.3	49.7	49.0

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

C. FOOD CONSUMPTION AND COMPOUND INTAKE

Mean food consumption was not affected by treatment in any group. Compound intake in male and female rats over the period of the study is shown in Table 5.3.1/01-2.

Table 5.3.1/01-2: Mean achieved dietary intake, in mg/kg bw/day of BCS-CN88460 in a rat 4-week dietary study

	BCS-CN88460, dietary concentration in ppm		
	300	1000	3000
Males	22.8	83.3	240
Females	25.6	86.5	285

D. HEMATOLOGY, CLINICAL CHEMISTRY, AND URINALYSIS

1. Hematology

In males at 3000 ppm, there was a decrease in total leucocyte counts and in absolute lymphocyte counts, as well as a slight increase in mean erythrocyte count. No effects on hematological parameters were observed at any dose in females or at 300 and 1000 ppm in males.

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Table 5.3.1/01-3: Selected hematological parameters in male and female rats administered BCS-CN88460 in the diet for four weeks

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Parameter	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
RBC 1012/L	8.962	8.648	8.992	9.576*	8.838	9.080	8.970	8.970
WBC 109/L	12.81	13.84	10.89	8.48*	8.62	9.48	7.58	8.51
Lymph. 109/L	10.71	11.06	8.86	6.68**	7.04	7.74	5.99	6.81

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

2. Clinical Chemistry

At 3000 ppm in males, total cholesterol, total protein, and albumin concentrations were increased in a statistically significant manner. Mean total bilirubin was statistically significantly decreased in all treated groups, in a largely dose-related manner.

Table 5.3.1/01-4: Selected clinical chemistry parameters in male and female rats administered BCS-CN88460 via the diet for four weeks

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Parameter	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Chol. mmol/L	1.686	1.833	1.520	2.132*	1.886	2.000	2.365	2.396
T. protein g/L	63.0	64.0	63.6	66.8**	60.0	61.8	67.3**	63.2
Albumin g/L	39.2	40.5	40.3	42.4**	39.0	39.2	42.8	39.6
Bilirubin µmol/L	0.78	0.63	0.26*	0.28*	1.22	0.68*	0.30**	0.22**

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

E. SACRIFICE AND PATHOLOGY

1. Terminal body weight and organ weights

Terminal body weight was decreased in males at 3000 ppm, but not in females at any dose level or in males at 300 or 1000 ppm.

At 3000 ppm in both males and females, both absolute and relative liver weights were increased relative to controls in a generally statistically significant manner, while absolute and relative thyroid weights were increased in males but not in females. At 1000 ppm, the increase in liver weight was statistically significant in females but not in males. Thyroid weight was not increased at 1000 ppm, and at 300 ppm there were no effects on the weight of either liver or thyroid in either males or females. Other organ weights were not affected by dietary administration of BCS-CN88460.

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Table 5.3.1/01-5: Terminal body weight and absolute and relative liver and thyroid weights in male and female rats administered BCS-CN88460 via the diet for four weeks

	BCS, dietary concentration in ppm			
	Males			
	0	300	1000	3000
Terminal body wt, g	381.6	393.3	374.1	328.2**
Liver wt, g	10.160	11.031	11.398	11.436
Liver to body wt, %	2.663	2.800	3.043*	3.483**
Liver to brain wt, %	493.027	559.107	571.349	584.967
Thyroid wt, g	0.01696	0.01818	0.01696	0.02050
Thyroid to body wt, %	0.00443	0.00465	0.00455	0.00623**
Thyroid to brain wt, %	0.82405	0.91842	0.85114	1.04697
	Females			
	0	300	1000	3000
Terminal body wt, g	208.4	208.2	207.9	210.0
Liver wt, g	5.223	5.486	6.629**	8.562**
Liver to body wt, %	2.510	2.634	3.263**	4.085**
Liver to brain wt, %	284.853	294.764	361.116**	459.389**
Thyroid wt, g	0.001158	0.01303	0.01348	0.01416
Thyroid to body wt, %	0.00555	0.00626	0.00648	0.00675
Thyroid to brain wt, %	0.63109	0.70101	0.72126	0.75898

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

2. Gross and microscopic pathology

At 3000 ppm, enlarged and / or dark liver were noted in some animals of both sexes, with prominent lobulation also noted in some females. At 1000 ppm, enlarged liver was noted in a few animals of both sexes, together with dark liver in some females. At 300 ppm, there were no treatment-related changes. One male showed enlarged liver, however as there were no corresponding effects on liver weight or on microscopic appearance, this observation was considered to be not treatment related.

Table 5.3.1/01-6: Incidence of macroscopic changes in the liver in male and female rats administered BCS-CN88460 via the diet for four weeks

	BCS, dietary concentration in ppm			
	Males			
	0	300	1000	3000
Terminal body wt, g	381.6	393.3	374.1	328.2**
Liver wt, g	10.160	11.031	11.398	11.436
Liver enlarged	0	1	2	2
Liver dark	0	0	0	3
Prominent lobulation of liver	0	0	0	0
	Females			
	0	300	1000	3000
Terminal body wt, g	208.4	208.2	207.9	210.0
Liver wt, g	5.223	5.486	6.629**	8.562**
Liver enlarged	0	0	1	5
Liver dark	0	0	3	2
Prominent lobulation of liver	0	0	0	3

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

At microscopic examination, the liver, thyroid, and kidney (males only) were observed to be target organs of BCS-CN88460. In the liver, the incidence of hepatocellular hypertrophy was increased in both males (3000 ppm) and females (1000 and 3000 ppm), while hepatocellular microvacuolation was increased in both males and females at 3000 ppm only.

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In the thyroid gland at 3000 ppm, treatment-related follicular cell hypertrophy was noted in both males and females. The single animal with follicular cell hypertrophy at 300 ppm in males, 1000 ppm in males, and 1000 ppm in females is considered to be not treatment-related as it was not associated with an incresae in the weight of the thyroid gland.. In the kidney, there was a dose-related increase in severity of tubular hyaline droplets in males at all doses; at 1000 and 3000 ppm only, there was also an increase in bilateral basophilic tubules, thus the observation of tubular hyaline droplets is only considered to be adverse at 1000 and 3000 ppm.

Table 5.3.1/01-7: Incidence of microscopic changes in the liver, thyroid, and kidney of male and female rats administered BCS-CN88460 via the diet for four weeks

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Parameter	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Liver, N examined	5	5	5	5	5	5	5	5
Hepatocellular hypertrophy	0	0	0	3	0	0	1	4
Hepatocellular microvacuolation	0	0	0	3	0	0	0	4
Thyroid, N examined	5	5	5	5	5	5	5	5
Follicular cell hypertrophy	0	1	1	5	0	0	1	3
Kidney, N examined	5	5	5	5	5	5	5	5
Basophilic tubules, bilateral								
Minimal	0	0	2	3	0	1	1	0
Slight	0	0	1	0	0	0	0	0
Total	0	0	3	3	0	1	1	0
Increased tubular hyaline droplets								
Minimal	0	2	2	1	0	1	0	0
Slight	0	1	3	1	0	0	0	0
Moderate	0	0	0	3	0	0	0	0
Total	0	3	5	5	0	1	0	0

Hyaline droplet nephropathy is a recognized lesion of the male kidney, related to the accumulation of α_{2u}-globulin in the proximal tubules of affected animals. This protein is naturally reabsorbed and accumulated in the renal tubular epithelium of the young male rat, however interaction between a chemical and α_{2u}-globulin can lead to its additional accumulation. As humans secrete α_{2u}-globulin in only trace amounts, this mechanism is generally accepted as being not relevant to humans. Thus, although the effects observed here in the kidney of male rats were considered to be treatment-related, they are considered not to be toxicologically relevant to man.

3. Quantification of alpha2u-globulin

In control animals, small alpha2u-globulin-positive droplets were observed in all males with a minimal severity. The severity of the observation of droplets staining positive for alpha2u-globulin increased in a dose-related manner at all doses. Additionally, the size and shape of the droplets were changed at 3000 ppm (moderately sized to large polyangular), and the number of affected tubules increased at 3000 ppm as well (up to more than 75%).

This clearly demonstrates the nature of the hyaline droplets observed in the kidney. A dose-related accumulation of alpha2u-globulin in the renal proximal tubules was observed, and was correlated with the higher incidence and severity of hyaline droplets in proximal tubules.

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Table 5.3.1/01-8: Incidence and mean severity of alpha2u-globulin accumulation in the kidney of male rats administered BCS-CN88460 via the diet for four weeks

	BCS-CN88460, dietary concentration in ppm			
	0	**300**	**1000**	**3000**
N examined	5	5	5	5
Alpha2u-globulin immunohistochemical staining				
Minimal	5	1	0	0
Slight	0	3	2	0
Moderate	0	0	2	1
Marked	0	0	1	4
Total	5	4	5	5
Mean severity	1.00	1.75	2.80	3.80

4. Intra-abdominal fat analysis

At 3000 ppm, only traces of BCS-CN88460, below the limit of validation of 50 µg/L, were detected in the intra-abdominal fat of 4/5 males and 4/5 females. At 1000 and 300 ppm, BCS-CN88460 was not detected in the intra-abdominal fat of either males or females.

5. Liver enzyme induction

There was no statistically significant effect on cytochrome P450 activity at 300 ppm in either males or females. At 1000 and 3000 ppm in males, moderate increases were noted for total cytochrome P450, EROD activity, and UDPGT-T4, with marked increases being observed for PROD, BROD, UDPGT-4-nitrophenol, and UDPGT-bilirubin. In females, no increase in total cytochrome P450 or in EROD activity was observed at any dose level. PROD was moderately increased in females, while BROD, UDPGT-4-nitrophenol, and UDPGT-T4 were markedly increased at 1000 and 3000 ppm. In females, UDPGT-bilirubin was increased at all doses. Enzyme induction in both males and females was increased in a dose-related manner.

A positive control study was conducted with phenobarbital, ß-naphthoflavone, and clofibrate administered to male and female rats by oral gavage for 28 days, in which the induction of total cytochrome P450 and of various Phase I and Phase II enzymes was measured. As shown in Table 5.3.1/01-9, the effect of BCS-CN88460 more closely resembles that of phenobarbital rather than either ß-naphthoflavone or clofibrate. This indicates that BCS-CN88460 is likely to be an activator of the constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR).

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Table 5.3.1/01-9: Liver enzyme activity in male and female rats administered BCS-CN88460 in the diet for four weeks, expressed as percent of controls

	BCS-CN88460, dietary ppm			Positive control data[1]		
	Males					
Parameter	**300**	**1000**	**3000**	**PB**	**ßNF**	**CLO**
Total cytochrome P450	101	124*	135**	205**	147**	139**
EROD	105	136*	157**	150	717**	92
PROD	85	161**	515**	1928**	209	230
BROD	129	256**	734**	4291***	411**	567**
UDPGT-4-nitrophenol	122	214**	247**	226**	300**	52
UDPGT-bilirubin	111	244**	317**	Not tested		
UDPGT-T4	102	147*	162**	Not tested		
	Females					
Parameter	**300**	**1000**	**3000**	**PB**	**ßNF**	**CLO**
Total cytochrome P450	86	89	92	181**	132**	109
EROD	100	112	128	132	905**	90
PROD	116	140	143	1249**	259	88
BROD	124	229**	524**	5491**	857**	400**
UDPGT-4-nitrophenol	107	198**	304**	202**	512**	116
UDPGT-bilirubin	167	250**	401**	Not tested		
UDPGT-T4	135	222**	319**	Not tested		

Statistically significant at * $p \leq 0.05$, ** $p \leq 0.01$; *** $p \leq 0.001$

[1] data obtained from [redacted], 2011; M-294727-01-2, in which animals were treated for 4 weeks with positive control substances phenobarbital (PB) at 75 mg/kg bw/day, ß-naphthoflavone (ßNF) at 75 mg/kg bw/day, or clofibrate (CLO) at 250 mg/kg bw/day

This activation of CAR and / or PXR is considered to be responsible for the treatment-related increase in plasma cholesterol in males and decrease in total bilirubin in both males and females, for the increases in liver and / or thyroid weight, and for the characteristic histopathological findings observed in those organs.

F. DEFICIENCIES

No specific deficiencies were noted in this study, which was not conducted in accordance to any guidelines. Toxicokinetic data were not collected because at the time the study was performed this was not a requirement nor was it a part of the standard study protocol. As summarized in the summary of the short-term toxicity section above, the toxicokinetic data obtained in the rat developmental toxicity study and at the 90-day timepoint of the chronic study can be used as a general indication of the behavior of BCS-CN88460 after oral administration.

III. CONCLUSIONS

BCS-CN88460 was found to be a potent inducer of cytochrome P450 3A and of the Phase II enzyme UDPGT, as well as a moderate inducer of cytochrome P450 2B in male rats, most likely through activation of the constitutive androstane receptor and / or the pregnane X receptor. Activation of these receptors is responsible for the increased liver and thyroid weight and the histopathological findings in these organs. The liver and thyroid, as well as the kidney in the male rat, were target organs after subacute dietary administration of BCS-CN88460 in the rat.

The dose level of 300 ppm (approximately 22.8 mg/kg bw/day in males and 25.6 mg/kg bw/day in females) was a NOAEL in male and female Wistar rats after dietary administration of BCS-CN88460 for 28 days.

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Report:	KCA 5.3.1/02; [redacted]; 2012; M-442490-01-1
Title:	BCS-CN88460 - Preliminary 28-day toxicity study in the mouse by dietary administration
Report No.:	SA 11309
Document No.:	M-442490-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Executive Summary

BCS-CN88460 was administered continuously via the diet to groups of C57BL/6J mice (five per sex per group) for at least 28 days at concentrations of 0, 200, 800, and 2000 ppm (equating to 0, 32, 133, and 330 mg/kg bw/day in males and 0, 41, 149, and 374 mg/kg bw/day in females, respectively). Animals were observed daily for mortality and clinical signs, a detailed physical examination was performed at least weekly, and body weight and food consumption were recorded on a weekly basis. Selected clinical chemistry parameters were determined at the end of the study. All animals were subjected to necropsy, selected organs were weighed, and a range of tissues were fixed and examined microscopically.

There were no treatment-related mortalities or clinical signs during the course of the study at any dose level in either sex.

At 2000 ppm (330 mg/kg bw/day in males and 374 mg/kg bw/day in females)

Mean body weight parameters and food consumption were unaffected by treatment in either sex throughout the study, except for a 69% decrease in mean cumulative body weight gain and a 17% decrease in mean food consumption in females during the first week of the study, compared to controls. Clinical chemistry assessment revealed higher mean aspartate aminotransferase, higher mean alanine aminotransferase, and slightly higher mean alkaline phosphatase activity, when compared to controls. In addition, total bilirubin concentrations were 53% lower in females when compared to controls.

At necropsy, mean absolute and relative liver weights were increased by 19-21% in males and 21-30% in females, when compared to the controls. Macroscopic evaluation revealed dark liver in 3/5 females. Microscopic evaluation revealed an increase in centrilobular hepatocellular hypertrophy in all males and in 4/5 females, hepatocellular necrotic foci in all males and all females, and focal hepatocellular single cell necrosis in all males and 4/5 females.

At 800 ppm (133 mg/kg bw/day in males and 149 mg/kg bw/day in females)

Clinical chemistry assessment revealed slightly higher mean aspartate aminotransferase activity in males only when compared to controls. In addition, total bilirubin concentrations were 58% lower in females when compared to controls, but this change was considered not to be an adverse effect of the test substance as lower total bilirubin concentrations do not represent any functional impairment in the test organism.

At necropsy, mean absolute and relative liver weights were incresaed by 10-14% in females, when compared to the controls. Microscopic examination revealed slight hepatocellular necrotic foci in 1/5 males and minimal focal hepatocellular single cell necrosis in 2/5 males.

At 200 ppm (32 mg/kg bw/day in males and 41 mg/kg bw/day in females)

No treatment-related effects were noted in either sex throughout the study, except that total bilirubin concentration was 44% lower in females when compared to controls. This change was considered not to be an adverse effect of the test substance, as lower total bilirubin concentrations do not represent any functional impairment in the test organism.

In conclusion, following continuous dietary administration of BCS-CN88460 to C57BL/6J mice for at least 28 days, based on the liver changes noted, the dietary concentration of 800 ppm (equating

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approximately to 149 mg/kg bw/day) was a No Observed Adverse Effect Level (NOAEL) in females, while the dietary concentratoin of 200 ppm (equating to approximately 32 mg/kg bw/day) was a No Observed Effect Level (NOEL) in males.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	White solid
Lot / Batch #:	NLL8674-19-4
Purity:	98.6%
CAS #	1255734-28-1
Stability of test compound:	Until 11 December 2012

2. Vehicle and / or positive control: none (dietary incorporation)

3. Test animals:	
Species:	mouse
Strain:	C57BL/6J
Age:	6-7 weeks at start of dosing
Weight at dosing:	21.0-23.7g (males); 16.0-18.6g (females)
Source:	[redacted]
Acclimation period:	14 days
Diet:	A04CP1-10 certified rodent powdered and irradiated diet
Water:	Filtered and softened water from municipal water supply
Housing:	Individually in suspended, stainless steel wire-mesh cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 air changes per hour
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN

1. In life dates: 18 April-31 May 2012

2. Animal assignment and treatment

During the acclimatization phase, all animals were weighed at least weekly and subjected to a detailed physical examination once. On the day of assignment to groups, all animals were weighed. An automatic randomization procedure (Pristima, version 6.3.2 build 17, Xybion Corp.) was used to select animals for the study from the middle of the weight range of the available animals, ensuring a similar body weight distribution among groups for each sex (± 20% of the mean body weight on the day of randomization). Animals were assigned permanent identification numbers within groups following randomization. Each animal was identified by a micro-identification implant.

Groups of 5 male and 5 female mice were given the vehicle control diet or the appropriate diet mixture at a constant ppm level. These dose levels were selected after evaluation of a 14-day dietary study in which BCS-CN88460 was administered via the diet to groups of 5 male and 5 female mice at concentrations of 1200, 2400, and 4200 ppm. From 2400 ppm, body weight gain was markedly decreased or body weight loss was observed, with slight decreases in food consumption. Absolute and relative liver weights were increased. At

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1200 ppm, there was no effect on body weight gain or food consumption, while absolute and relative liver weights were increased in both males and females.

Based on these results, the concentrations selected for the present 28-day study were 0, 200, 800, and 2000 pm.

3. Diet preparation and analysis

The test item was incorporated into the diet by dry mixing to provide the required dietary concentrations. There was one preparation for each concentration. When not in use, the diet formulations were stored at room temperature.

Dietary levels of the test item were verified for each concentration. The stability of the test item in the diet was determined separately during the course of the current study at concentrations of 60 and 10000 ppm, and was demonstrated to be acceptable under storage and usage conditions similar to those of the current study. Measured concentrations of study formulations ranged from 91% to 97% of nominal concentrations, and were thus within the in-house target ranges (85-115%).

4. Statistics

Mean and standard deviation were calculated for each group, and all statistical analyses were carried out separately for males and females.

For body weight gain, terminal body weight, absolute and relative organ weights, and clinical chemistry parameters, mean and standard deviation were calculated for each group, and per time period for body weight gain / day parameters. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight and average food consumption per day, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on log-transformed data. If the ANOVA on log-transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on log-transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the

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Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the man of the control group using the two-sided Dunn test.

C. METHODS

1. Observations

Animals were checked for moribundity and mortality twice daily on weekdays, and once daily on weekends or public holidays, and were observed for clinical signs at least once daily. Detailed physical examinations were performed at least weekly during the treatment period. 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 item administration, and then at weekly intervals throughout the treatment period. At scheduled necropsy, terminal body weight was collected by weighing animals which had been diet-fasted overnight.

3. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals in all groups during the study. Any food spillage was noted. The weekly mean achieved dosage intake in mg/kg bw/day for each week and for weeks 1-4 was calculated for each sex.

4. Ophthalmoscopic examination

Ophthalmoscopic examination was not conducted.

5. Clinical chemistry

On study day 30, 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 were anesthetized by inhalation of isoflurane. Blood was collected on clot activator for serum clinical chemistry determinations.

6. Urinalysis

Urinalysis was not conducted.

7. Sacrifice and pathology

On study day 30, all animals from all groups were anesthetized by inhalation of isoflurane and then exsanguinated. An approximately equal number of animals randomly distributed among all groups were sacrificed on each day. Animals were diet fasted overnight prior to sacrifice. All animals were necropsied, including the examination of all major organs, tissues, and body cavities. Microscopic abnormalities were recorded, sample, and examined microscopically.

At final sacrifice, weights were taken (paired organs were weighed together) of adrenal gland, brain, kidney, liver, ovary, spleen, testis, and uterus including cervix. The adrenal gland, epididymis, kidney, liver, ovary, pituitary gland, spleen, testis, thyroid gland with parathyroid gland, uterus including cervix, vagina, and any macroscopic findings were sampled and fixed by immersion in neutral buffered 10% formalin. Testis and epididymis were fixed in Davidson's fixative. Histological sections of these organs were prepared for all animals in all groups and stained with hematoxylin and eosin.

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Histopathological examinations of all sampled organs (except parathyroid gland) were performed on all slides from all animals from control and high dose groups. Kidney, liver, thyroid gland, and macroscopic findings of all animals were examined in the intermediate dose groups.

II. RESULTS AND DISCUSSION

A. CLINICAL SIGNS AND MORTALITY

1. Clinical signs of toxicity

There were no treatment-related clinical signs observed during the study at any dose level in either sex.

2. Mortality

There were no mortalities during the study.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 2000 ppm in females, between study days 1 and 8, mean cumulative body weight gain was decreased by 69%, although this decrease was not statistically significant. There were no effects on either body weight or body weight gain in males at any dose, or in females at 200 or 800 ppm.

Table 5.3.1/02-1: Mean body weight and body weight gain in male and female rats administered BCS-CN88460 via the diet for four weeks

		BCS-CN88460, dietary concentration in ppm							
		Males				Females			
	Day	0	200	800	2000	0	200	800	2000
Body wt, g	1	22.10	22.32	22.38	21.90	17.62	17.26	17.48	17.84
	8	22.70	23.24	23.28	22.66	18.78	18.40	18.32	18.20
	15	23.30	23.76	24.32	23.50	19.28	19.10	19.04	19.72
	22	23.44	24.36	24.26	23.48	19.44	19.72	19.40	19.84
	29	24.32	25.08	25.30	24.76	20.40	20.30	20.40	21.00
Body wt gain, g	1-29	2.22	2.76	2.92	2.86	2.78	3.04	2.92	3.16

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

C. FOOD CONSUMPTION AND COMPOUND INTAKE

In females at 2000 ppm, there was a slight reduction in food consumption between study day 1 and study day 8. All other food consumption measures were unaffected by treatment. Compound intake in male and female mice over the period of the study is shown in Table 5.3.1/02-2.

Table 5.3.1/02-2: Mean achieved dietary intake, in mg/kg bw/day of BCS-CN88460 in a mouse 4-week dietary study

	BCS-CN88460, dietary concentration in ppm		
	200	800	2000
Males	32	133	330
Females	41	149	374

D. OPHTHALMOSCOPIC EXAMINATION

Ophthalmoscopic examination was not conducted in this study.

E. HEMATOLOGY, CLINICAL CHEMISTRY, AND URINALYSIS

1. Hematology

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Hematological analysis was not conducted in this study.

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2. Clinical Chemistry

Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) activities were increased in a biologically significant manner in both males and females at 2000 ppm, along with slightly increased alkaline phosphate (AP) activity in males only. At 800 ppm, aspartate aminotransferase was increased in a statistically non-significant manner. Total bilirubin was decreased in females at all dietary levels. This is not considered to be an adverse effect of the test substance but does indicate a biological effect of the test substance.

Table 5.3.1/02-3: Selected clinical chemistry parameters in male and female rats administered BCS-CN88460 via the diet for four weeks

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Parameter	**0**	**200**	**800**	**2000**	**0**	**200**	**800**	**2000**
Bilirubin µmol/L	0.62	0.84	0.64	0.56	1.10	0.62*	0.46**	0.52**
ASAT IU/L	89.8	86.2	133.2	196.8*	108.6	125.0	109.2	148.2
ALAT IU/L	34.5	32.0	42.4	159.0*	35.6	36.2	37.4	74.8*
AP IU/L	110.6	102.4	125.0	131.4*	178.6	181.4	181.0	183.2

Statistically significant at * $p \leq 0.05$, ** $p \leq 0.01$; *** $p \leq 0.001$

3. Urinalysis

Urinalysis was not conducted in this study.

F. SACRIFICE AND PATHOLOGY

1. Terminal body weights and organ weight

Terminal body weight was unaffected by dietary administration of BCS-CN88460 in either males or females. Absolute and relative liver weights were statistically significantly increased at 2000 ppm in both males and females. Although it was not statistically significant, there was a 10-14% increase in absolute and relative liver weights in female mice at 800 ppm.

Table 5.3.1/02-4: Terminal body weight and absolute and relative liver weights in male and female mice administered BCS-CN88460 via the diet for four weeks

	BCS, dietary concentration in ppm			
	Males			
	0	**200**	**800**	**2000**
Terminal body wt, g	19.96	20.56	20.48	20.14
Liver wt, g	0.893	0.906	0.965	1.073**
Liver to body wt, %	4.472	4.413	4.721	5.325**
Liver to brain wt, %	200.824	202.746	213.691	242.236**
	Females			
	0	**200**	**800**	**2000**
Terminal body wt, g	16.16	16.20	16.40	16.74
Liver wt, g	0.723	0.761	0.805	0.938**
Liver to body wt, %	4.475	4.698	4.908	5.593**
Liver to brain wt, %	161.447	170.231	183.908	206.803**

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

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2. Gross and microscopic pathology

At 2000 ppm, dark liver was noted in 3 of 5 females. The incidence of this observation in one male at 800 ppm and one female at 200 ppm was considered to be incidental as it was not associated with an increase in liver weight or with related histopathological findings.

Table 5.3.1/02-5: Incidence of macroscopic changes in the liver in male and female mice administered BCS-CN88460 via the diet for four weeks

	BCS, dietary concentration in ppm			
	Males			
	0	**200**	**800**	**2000**
Terminal body wt, g	19.96	20.56	20.48	20.14
Liver wt, g	0.893	0.905	0.965	1.073**
Liver dark	0	0	1	0
	Females			
	0	**200**	**800**	**2000**
Terminal body wt, g	16.16	16.20	16.40	16.74
Liver wt, g	0.723	0.761	0.805	0.938**
Liver dark	0	1	0	3

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

At microscopic examination, the incidence of centrilobular hepatocellular hypertrophy, hepatocellular necrotic foci, and focal hepatocellular single cell necrosis were noted in both males and females at 2000 ppm. The incidence of hepatocellular necrotic foci and focal hepatocellular single cell necrosis was also increased in males at 800 ppm. There were no histopathological findings noted in females at 800 ppm or in either males or females at 200 ppm.

Table 5.3.1/02-6: Incidence of microscopic changes in the liver of male and female mice administered BCS-CN88460 via the diet for four weeks

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Parameter	**0**	**200**	**800**	**2000**	**0**	**200**	**800**	**2000**
Liver, N examined	5	5	5	5	5	5	5	5
Hepatocellular hypertrophy	0	0	0	5	0	0	0	4
Hepatocellular necrotic focus(i)	0	0	1	5	0	0	0	5
Hepatocellular single cell necrosis	0	0	2	5	0	0	0	4

G. DEFICIENCIES

No specific deficiencies were noted in the study which was not conducted in accordance to any guidelines. Toxicokinetic data was not collected because at the time the study was performed this was not a requirement nor was it a part of the standard study protocol. As summarized in the summary of the short-term toxicity section above, the toxicokinetic data obtained in the rat developmental toxicity study and at the 90-day timepoint of the chronic study can be used as a general indication of the behavior of BCS-CN88460 after oral administration.

III. CONCLUSIONS

Effects of BCS-CN88460 after subacute dietary administration to male and female mice were limited to the liver. Observations which were increased in either a statistically or biologically significant manner were increased liver weight, increased aspartate and alanine aminotransferase activities as well as alkaline phosphatase activity, and in females only decreased bilirubin, and in both sexes an increase in the incidence of hepatocellular hypertrophy, hepatocellular necrotic foci, and hepatocellular single cell necrosis.

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Based on these observations in the liver, the dose level of 800 ppm (approximately 149 mg/kg bw/day) was an NOAEL in females, while the dose level of 200 ppm (approximately 32 mg/kg bw/day) was an NOEL in males.

Report:	KCA 5.3.1/03; [redacted]; 2014; M-503716-01-1
Title:	BCS-CN88460 - Preliminary 28-day toxicity study in the dog by dietary administration
Report No.:	SA 12107
Document No.:	M-503716-01-1
Guideline(s):	US EPA OCSPP 870.SUPP
Guideline deviation(s):	not specified
GLP/GEP:	**no**

Executive Summary

BCS-CN88460 was administered to male and female beagle dogs by dietary administration. Groups of two animals per sex received the test item at dietary concentrations of 0, 300, 1000, or 3000 ppm (equating to 0, 12.7, 37.7, and 76.9 mg/kg bw/day in males and 0, 11.3, 36.5, and 90.2 mg/kg bw/day in females) for 28 days. Each animal was checked for ill health, moribundity, and mortality twice daily or once daily on weekends and public holidays during the acclimatization phase and throughout the study. Food consumption was recorded daily throughout the study, and body weight was measured weekly and prior to necropsy. Additionally, a detailed clinical examination was performed approximately every week prior to treatment and during the treatment period. Once during the acclimatization phase and at the end of treatment, ophthalmological examination, blood analysis (hematology and clinical chemistry), and urinalysis were performed. All animals were subjected to a detailed necropsy. Selected organs were weighted and a range of tissues were taken and processed for histopathological examination.

Dietary administration of BCS-CN88460 to male and female beagle dogs at 300, 1000, and 3000 ppm did not cause any mortalities, and did not induce any changes at the physical and ophthalmological examinations, hematology investigation, or urinalysis. Plasma concentrations of the test item at the end of the study showed a dose-related increase, with a maximum concentration measured 2 or 4 hours after food distribution.

<u>At 3000 ppm (equating to 76.9 and 90.2 mg/kg bw/day in males and females, respectively)</u>

At clinical observation, increased salivation was observed on two occasions for one male, and wasted appearance was noted for one female on day 8. At the end of the study, 3/4 animals lost weight (-0.2 to -0.5 kg), and the fourth animal of the group only gained 0.1 kg compared to a body weight gain of 0.6 to 1.2 kg in controls. Food consumtpion values were lower for almost all animals compared to controls or to their own pre-test value.

In clinical chemistry, increased alkaline phosphatase activity was observed in one male and two females compared to their own pre-test value. In addition, lower total cholesterol concentrations were observed in one male and one female compared to their own pre-test value.

At scheduled sacrifice, terminal body weight of females was 16% lower than controls. Mean absolute and relative liver weights were 26-52% higher in both sexes compared to controls. The macroscopic examination revealed an enlarged liver in one male. At the microscopic examination, centrilobular to panlobular hepatocellular hypertrophy and decreases in hepatocellular glycogen accumulation was noted in the liver of both sexes, together with an increase in brown pigmen accumulation in Kupffer cells in females.

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At 1000 ppm (equating to 37.7 and 36.5 mg/kg bw/day in males and females, respectively)
No treatment-related changes were observed in males. In females, the only treatment-related change was an increase in alkaline phosphatase activity in one animal relative to its pre-study value. This change was considered not to be adverse, as it was noted in isolation and was not associated with microscopic changes in the liver.

At 300 ppm (equating to 12.7 and 11.3 mg/kg bw/day in males and females, respectively)
No treatment-related changes were observed in either sex.

In conclusion, a dietary level of 1000 ppm administered to beagle dogs for at least 28 days was considered to be a No Observed Effect Level (NOEL) in males and a No Observed Adverse Effect Level (NOAEL) in females.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	White solid
Lot / Batch #:	NLL 8674-28-2
Purity:	98.0%
CAS #	1255734-28-1
Stability of test compound:	Until 5 December 2013

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	dog
Strain:	beagle
Age:	Approximately 8 months
Weight at dosing:	6.0-8.0kg (males), 5.6-6.0kg (females)
Source:	[redacted]
Acclimation period:	At least 5 weeks
Diet:	Certified canine meal 12563-P1
Water:	Filtered and softened water from the municipal water supply, available ad libitum
Housing:	Individually in stainless steel kennels; when possible, pair-housed overnight
Environmental conditions:	
Temperature:	18-21°C
Humidity:	40-70%
Air changes:	Target of 15 air changes per hour
Photoperiod:	12 hours dark, 12 hours light

B. STUDY DESIGN

1. In life dates: 6 February-4 April 2013

2. Animal assignment and treatment

During the acclimatization phase, animals where weighed at least weekly before food distribution, and food intake was measured for a minimum of 5 consecutive days before start of treatment. All animals during the acclimatization phase were subjected to ophthalmological examination, hematology and clinical chemistry investigations, and urinalysis. Animals were selected and randomized for use in the study based on acceptable findings from physical and ophthalmological examinations, body weight, food consumption,

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clinical chemistry and hematology analyses, and urinalysis. Animals were allocated to dosage groups in order to ensure a similar body weight distribution among groups of each sex, while ensuring full siblings were not placed in the same treatment group.

On each treatment day, approximately 330 grams of diet containing the test item at the appropriate concentration, moistened with 470 grams of water at the time of distribution, was given to each animal for about 1.5 hours in the morning.

Dietary concentrations of BCS-CN88460 were 0, 300, 1000, and 3000 ppm.

3. Diet preparation and analysis

The test item BCS-CN88460 was ground to a find powder prior to use. The appropriate amount, on a weight/weight basis, of test item was incorporated into the ground diet to provide the required dietary concentrations of 0, 300, 1000, and 3000 ppm. One preparation was performed to provide the amount of treated diet required for the study. Each test diet mixture was stored at room temperature.

The stability of BCS-CN88460 in ground canine diet was demonstrated in a separate study for up to 66 days under frozen storage conditions followed by 10 days at room temperature, or after at least 76 days of storage at room temperature. Once moistened, BCS-CN88460 was stable for at least 4 hours at room temperature. The homogeneity of the test item in the diet was verified before the start of the study at 300 and 3000 ppm on the preparation, to demonstrate adequate formulation procedure. The dietary level of the test item in the diet was verified at each concentration. The mean values obtained from the homogeneity check were used as measured concentrations.

The homogeneity and concentration results ranged from 88% to 101% of the nominal concentrations, and the formulations were thus considered acceptable for the study.

4. Statistics

Due to the small number of animals per sex in each group, mean values and standard deviations were not calculated for the quantitative parameters, with the exception of the achieved intake values, where individual and mean values are presented. In addition, mean and standard deviation were calculated for the liver weight. Where applicable, the results obtained at the end of the treatment period were compared individually for each animal with the pre-study values, each animal serving as its own control.

C. METHODS

1. Observations

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

Observed clinical signs were recorded at least once daily throughout the study. Any deviation from normal was recorded in respect to the nature and severity of the observation. Daily examination of the kennels for vomit, diarrhea, or blood was also conducted.

An additional detailed clinical examination was performed in an open area approximately weekly throughout the study. Recording of clinical signs included but were not limited to changes in general behavior and appearance, skin and fur, teeth and gum, eyes, ears, mucous membranes, gait, posture, and response to handling.

At the end of the study, a detailed physical examination was performed on all dogs, and included but was not restricted to examination of fur and skin, eyes, ears, teeth, gums,

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mucous membranes, rectal temperature, gait, stance, general behavior, chest including heart and respiratory rate, abdomen including palpation, external genitalia, and mammary glands. Additional parameters evaluated during this examination included mental state (level of consciousness and behavioral change), posture, gait and motor function, muscle tone, postural reaction (tactile and visual place, conscious proprioceptive positioning such as knuckling, hopping, and wheelbarrowing), spinal nerve reflexes (patellar, flexor withdrawal, pelvic and thoracic limb, perianal, panniculus), sensation to superficial and deep pain, and cranial nerve reflexes (general examination of the head, direct and indirect pupillary light, palpebral (blink and corneal), and menace.

2. **Body weight**
Body weights were measured prior to feeding at least weekly during the acclimatization phase and treatment period, and terminal body weight was measured before final necropsy.

3. **Food consumption and compound intake**
Food intake was measured for a minimum of five consecutive days before the start of treatment. The weight of food supplied to each animal and that remaining were recorded daily throughout the treatment period. From these records, the mean weekly consumption was recorded for each dog. Food spillage was also recorded.

The individual and group mean achieved dosage for each sex, expressed as mg/kg bw/day, was calculated for each week and the overall mean was derived.

4. **Ophthalmoscopic examination**
During the acclimatization phase and again at the end of treatment, all animals were subjected to an ophthalmological examination after instillation of an atropinic agent. Each eye was examined by means of an indirect ophthalmoscope. In the case of any treatment-related effects, photographic records were made of the affected animals and kept in the study files.

5. **Hematology and clinical chemistry**
On pre-test day 20 and on study day 25, blood samples were taken from all animals in all groups by puncture of the jugular vein. Blood was collected on EDTA for hematology, clot activator for serum clinical chemistry, and sodium citrate for coagulation parameters. Blood smears were prepared and stained using May-Gruenwald-Giemsa method, and examined if the hematological parameters were abnormal. Any significant change in the general appearance of the serum was recorded during measurement of clinical chemistry parameters.

6. **Urinalysis**
On pre-test day 21 and on study day 23 in the morning, overnight urine samples were collected from all animals in all groups. Access to water was restricted during urine collection. Any significant change in the general appearance of the urine was recorded, urine samples were weighed to determine urinary volume, and urinary refractive index was measured. Quantitative and semi-quantitative parameters were determined, and microscopic examination of the urinary sediment was performed after centrifugation of the urine.

7. **Bioanalytical examination**
At the end of the study, a blood sample was collected from the jugular vein into heparinized tubes for all treated animals prior to food distribution and then at 1, 2, and 4 hours after food distribution. Blood samples were also collected in the same way from the control animals prior to food administration. Plasma was prepared from blood collected into heparinized vials, for determination of the concentration of the test item and potentially its major metabolites. Samples were stored in the dark at approximately -20°C until analysis by HPLC with tandem mass spectrometry.

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8. Sacrifice and pathology

On study days 30 and 31, all surviving animals from all groups were tranquilized with intramuscular injection of acepromazine and sacrificed by exsanguination under deep anesthesia ensured by intravenous injection of pentobarbital.

All animals either found dead or killed for humane reasons were necropsied, including an examination of external surfaces, all orifices, and all major organs, tissues, and body cavities. Macroscopic abnormalities were recorded, sampled, and examined microscopically.

From all animals, the adrenal gland, brain, epididymis, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland with parathyroid, and uterus including cervix were weighed fresh. Paired organs were weighed together. Organs and tissues were sampled, and samples were fixed by immersion in 10% neutral buffered formalin, with the exception of the eye, optic nerve, epididymis, and testis, which were fixed in Davidson's fixative. After fixation, tissue samples were embedded in paraffin wax (with the exception of the larynx and pharynx), and histological sections were prepared and examined from all the animals in all groups.

II. RESULTS AND DISCUSSION

A. CLINICAL SIGNS AND MORTALITY

1. Clinical signs of toxicity

At 3000 ppm, one male showed increased salivation days 15 and 22, and wasted appearance was noted for one female on day 8. No treatment-related clinical signs were observed at either 300 or 1000 ppm.

No changes were observed in any treated groups at physical examination, and rectal temperature was not affected by treatment.

2. Mortality

No mortalities occurred during the study.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 3000 ppm, both males and one female lost weight during the study, while the remaining female in this group had almost static body weight over the study. Body weight losses ranged from 0.2 to 0.7 kg, compared to a body weight gain of 0.6 to 1.2 kg in control animals. Body weight was unaffected at 300 and 1000 ppm.

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Table 5.3.1/03-1: Individual body weight and body weight gain in male and female beagle dogs administered BCS-CN88460 via the diet for four weeks

BCS-CN88460, ppm	Sex	Animal	Study day					Body wt gain, kg
			1	8	15	22	29	1-29
0	M	0312	6.0	6.2	6.6	6.9	7.2	1.2
		0313	6.5	7.0	7.0	7.0	7.4	0.9
	F	0314	5.8	6.1	6.3	6.7	6.8	1.0
		0315	5.7	5.8	6.0	6.2	6.3	0.6
300	M	0316	6.9	7.1	7.4	7.5	7.9	1.0
		0317	6.1	6.5	6.6	6.8	7.0	0.9
	F	0318	5.8	6.1	5.9	6.1	6.4	0.6
		0319	5.6	5.8	5.8	6.2	6.0	0.4
1000	M	0320	6.3	6.4	6.5	6.5	6.7	0.4
		0321	7.8	8.0	8.3	8.5	8.7	0.9
	F	0322	5.8	5.8	5.8	6.1	6.2	0.4
		0323	6.0	6.2	6.2	6.4	6.4	0.4
3000	M	0324	6.6	6.4	6.2	6.1	6.1	-0.5
		0325	8.0	7.8	7.6	7.7	7.8	-0.2
	F	0326	5.8	5.7	5.8	5.8	5.9	0.1
		0327	5.6	5.3	5.1	5.3	5.3	-0.3

C. FOOD CONSUMPTION AND COMPOUND INTAKE

At 3000 ppm, food consumption was decreased compared to controls or to each animal's own pre-study value for three of the four animals. For female 0326, however, food consumption during the study was similar to that observed prior to the start of the study. This is reflected in the body weight loss of three animals and the slight body weight gain in female 0326.

The achieved dosages at 300, 1000, and 3000 ppm were 12.7, 37.7, and 76.9 mg/kg bw/day in males, and 11.3, 36.5, and 90.2 mg/kg bw/day in females.

D. OPHTHALMOSCOPIC EXAMINATION

No abnormalities were noted at the ophthalmological examination in any animal.

E. HEMATOLOGY, CLINICAL CHEMISTRY, AND URINALYSIS

1. Hematology

No treatment-related changes were noted during the study.

2. Clinical Chemistry

At 3000 ppm, alkaline phosphatase was increased in one of the two males and in both females, when compared to their own pre-study value. Total cholesterol concentrations were decreased in one male and one female at this dose.

At 1000 ppm, one female showed increased alkaline phosphatase compared to her pre-study value. However, no histopathological changes were observed in that animal and thus this finding was considered not to be adverse.

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Table 5.3.1/03-2: Selected clinical chemistry parameters in male and female beagle dogs administered BCS-CN88460 via the diet for four weeks

BCS-CN88460, ppm	Sex	Animal	Alkaline phosphatase, IU/L		Cholesterol, mmol/L	
			Pre-study day 20	Study day 25	Pre-study day 20	Study Day 25
0	M	0312	110	119	2.99	2.75
		0313	130	117	2.75	3.34
	F	0314	129	123	2.91	2.51
		0315	97	115	2.68	2.86
300	M	0316	107	138	2.99	3.08
		0317	298	280	1.89	2.14
	F	0318	141	176	3.45	3.42
		0319	132	171	3.70	4.43
1000	M	0320	131	157	2.74	2.86
		0321	117	171	3.34	3.05
	F	0322	133	384	2.69	2.20
		0323	121	157	3.73	3.80
3000	M	0324	227	255	3.20	1.93
		0325	217	372	3.07	3.13
	F	0326	100	555	2.92	2.36
		0327	124	361	2.85	1.90

3. Urinalysis

No treatment-related changes were noted for any urinalysis parameters in either males or females.

3. Bioanalytical measurements

At the end of the study, the concentration of BCS-CN88460 in the plasma was measured in the plasma of treated animals both before and at various time points after food distribution.

Table 5.3.1/03-3: Concentration of BCS-CN88460, in mg/L in the plasma of male and female dogs administered BCS-CN88460 via the diet for 28 days

Sex	Diet conc., ppm	Animal ID	Time after food distribution			
			0 hours	1 hour	2 hours	4 hours
M	0	1M0312	< LOQ	NS	NS	NS
		1M0313	< LOQ	NS	NS	NS
	300	2M0316	< LOQ	0.111	0.180	0.230
		2M0317	< LOQ	0.0555	0.0726	0.0563
	1000	3M0320	0.0164	0.529	0.919	0.965
		3M0321	0.0157	0.707	1.48	1.60
	3000	4M0324	0.0226	2.71	3.25	2.89
		4M0325	0.0187	2.52	3.36	3.36
F	0	1F0314	< LOQ	NS	NS	NS
		1F0315	< LOQ	NS	NS	NS
	300	2F0318	< LOQ	0.127	0.188	0.240
		2F0319	< LOQ	0.0206	0.0336	0.0546
	1000	3F0322	< LOQ	0.180	0.589	0.631
		3F0323	< LOQ	NS	0.164	0.167
	3000	4F0326	< LOQ	1.37	1.87	1.57
		4F0327	< LOQ	0.907	1.80	1.44

< LOQ = below limit of quantification (0.01 mg/L)
NS = no sample

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F. SACRIFICE AND PATHOLOGY

1. Terminal body weight and organ weight

At 3000 ppm, terminal body weight was decreased in females, while absolute and relative liver weights were increased in both males and females.

Table 5.3.1/03-4: Terminal body weight and absolute and relative liver weight in male and female dogs administered BCS-CN88460 via the diet for 28 days

		BCS-CN88460, dietary concentration in ppm							
		Males				Females			
	Animal	0	300	1000	3000	0	300	1000	3000
Terminal body wt, kg	A	7.1	7.9	6.6	6.0	6.8	6.3	6.0	5.8
	B	7.2	6.9	8.7	7.7	6.1	6.1	6.5	5.1
Absolute liver weight, g		254.3	263.3	282.9	320.5	191.2	228.7	257.9	244.7
Liver wt, % body wt		3.56	3.55	3.68	4.78	2.96	3.69	4.12	4.50
Liver wt, % brain wt		321.1	377.2	419.2	467.9	289.0	338.1	392.6	368.3

2. Gross pathology

At 3000 ppm, one male showed enlarged liver. No other treatment-related changes were noted.

2. Microscopic pathology

At 3000 ppm in the liver, centrilobular to panlobular hepatocellular hypertrophy and decreased severity of hepatocellular glycogen accumulation were noted in both sexes. In females only, brown pigments in Kupffer cells were also noted. At 1000 and 300 ppm, there were no treatment-related changes at microscopic examination.

Table 5.3.1/03-5: Microscopic pathology in livers of male and female dogs administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	300	1000	3000	0	300	1000	3000
Hepatocellular glycogen accumulation, diffuse								
Minimal	0	0	1	2	0	0	1	1
Slight	2	2	1	0	1	2	1	0
Moderate	0	0	0	0	1	0	0	0
Total	2	2	2	2	2	2	2	1
Hepatocellular hypertrophy, centrilobular to panlobular								
Minimal	0	0	0	2	0	0	0	1
Accumulation of brown pigment in Kupffer cells								
Minimal	0	0	0	0	0	0	0	2
Total	0	0	0	0	0	0	0	2

G. DEFICIENCIES

No specific deficiencies were noted.

III. CONCLUSIONS

Administration of BCS-CN88460 to male and female beagle dogs caused treatment-related effects in the liver, including increased organ weight, hepatocellular centrilobular to panlobular hypertrophy, and decreased hepatocellular glycogen accumulation. Body weight and body weight gain were decreased in both males and females. The dietary level of 1000 ppm was observed to be a NOEL in males and an NOAEL in females.

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CA 5.3.2 Oral 90-day study

Report:	KCA 5.3.2/01; [redacted]; 2017; M-487478-02-1
Title:	BCS-CN88460 - 90-day toxicity study in the rat by dietary administration - Final report amendment no. 1
Report No.:	M-487478-02-1
Document No.:	M-487478-02-1
Guideline(s):	OECD guideline 408 (September, 1998) EEC Directive 2001/59/EC, Method B.26 (August, 2001) US EPA OCSPP guideline number 870.3100 MAFF in Japan Notification 12 Nousan n°8147 (November, 2000)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

BCS-CN88460 was administered via the diet to groups of Wistar rats (10 per sex per group) at concentrations of 0, 100, 300, and 1000 ppm (equating approximately to 0, 6.34, 18.4, and 63.5 mg/kg bw/day in males and 0, 7.92, 21.9, and 80.9 mg/kg bw/day in females) for at least 90 days. In addition, an additional 10 animals per sex were added to the control and high dose groups to assess the reversibility of any effects observed at the high dose. These animals were fed either control or test diet for at least 90 days, and were then maintained during the recovery phase on untreated control diet for one month following the termination of the three-month exposure period. Animals were observed daily for mortality and for clinical signs, and body weight was measured weekly. Food consumption was measured twice weekly during the first six weeks of treatment and weekly thereafter. A detailed physical examination was performed once during the acclimatization phase and weekly throughout the study. All surviving animals of the main study group (dosing phase) were subjected to a neurotoxicity assessment) spontaneous motor activity, fore- and hindlimb grip strength, landing foot splay, and rectal temperature) during week 11 of the study. Ophthalmological examinations were performed on all animals during the acclimatization phase and on all animals of the control and high dose groups during week 13. Urine samples were collected overnight on the week before scheduled necropsy from all animals. Before scheduled necropsy, a blood sample was collected from the retro-orbital venous plexus of each animal for hematology and clinical chemistry determinations. All animals were necropsied, selected organs were weighed, and a range of tissues were taken, fixed, and examined microscopically.

Up to the highest dietary concentration of 1000 ppm, dietary administration of BCS-CN88460 induced no mortalities, and no treatment-related changes were observed on food consumption or in the hematology, clinical chemistry, physical, ophthalmological, or neurological examinations.

At 1000 ppm (63.5 and 80.9 mg/kg bw/day in males and females, respectively)

Mean body weight parameters were affected by treatment in both sexes. In males, mean body weight gain per day was reduced on several occasions during the study when compared to controls. Mean absolute body weight gain was reduced by between 8 and 13% throughout the study, and mean body weight was slightly lower than the controls by up to 8%. At the end of the treatment period, the overall mean body weight gain and mean body weight were 12% and 7% decreased compared to controls. In females, mean body weight gain per day and mean absolute body weight gain were reduced by 29% on day 8. Thereafter, mean body weight gain per day was similar to controls, while mean absolute body weight gain remained statistically significantly reduced during the first three weeks due to the initial effect on body weight. At the end of the treatment period, the overall mean body weight gain was 8% lower than the controls, while mean body weight was similar to controls.

Clinical chemistry determinations showed lower mean total bilirubin concentrations in both sexes compared to controls. A slightly higher mean total cholesterol concentration was also noted in females in comparison to controls.

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Urinalysis revealed cellular casts in the urine of 5/10 males. As this finding was associated with relevant microscopic changes in the kidney, it was considered to be treatment-related.

At necropsy, compared to controls, mean terminal body weight was lower in males and females. Mean liver and body weight ratio was 11% higher in males. This change was considered to be related to the lower mean terminal body weight in this group. Mean absolute and relative liver weights were 18-28% higher in females. These changes were associated with relevant microscopic findings and were considered to be treatment-related. Mean thyroid gland to body weight ratio was 21% higher in males, and was considered to be related to treatment as it was associated with relevant microscopic findings. Mean kidney to body weight ratio was 15% higher in males. This change was considered to be related to the decreased mean terminal body weight. Gross pathology examination revealed enlarged liver in 8/10 females, which was correlated to microscopic hepatocellular hypertrophy in 6/8 females and was therefore considered to be treatment-related.

Microscopic examiantion showed treatment-related findings in the liver, kidney, and thyroid gland. In the liver, minimal to slight periportal to panlobular hepatocellular hypertrophy was observed in 6/10 females. In the kidney, a higher incidence and severity of hyaline droplets in proximal tubules and bilateral basophilic tubules were observed in males, together with minimal granular casts for some males. In the thyroid gland, a higher incidence of minimal follicular cell hypertrophy and of colloid alteration was observed in both sexes.

In the recovery phase, the mean body weight of high dose males remained 8-10% lower than the control group. The cumulative body weight gain for high dose male rats was higher than the control group during the first three weeks of the recovery period, and at the end of the recovery period the overall mean body weight gain in males was 79% higher compared to the controls. In high dose females, mean body weight parameters were essentially comparable to controls.

Treatment-related changes noted at the end of the treatment phase in clinical chemistry and urinalysis parameters were considered to be reversible, as the differences from controls were not seen at the end of the recovery phase.

At necropsy, compared to controls, mean terminal body weight was lower in males, and mean liver to body weight ratio was increased in males. This change was considered to be related to the decrease in mean terminal body weight. The treatment-related changes observed at the end of the treatment period, i.e. liver weight changes in females and thyroid gland weight changes in males, were not observed at the end of the recovery period and were thus considered to be reversible. Gross pathology examination revealed no treatment-related macroscopic findings, thus the treatment-related findings observed at the end of the treatment period were also considered to be reversible.

Microscopic examination at the end of the recovery period showed that the treatment-related findings observed at the end of the treatment phase in the liver, kidney, and thyroid gland were reversed, with the exception of an increased incidence and severity of bilateral basophilicu tubules and the presence of granular cast(s) still observed in the kidney of males, and a slightly higher incidence of colloid alteration still observed in the thyroid gland of males.

<u>At 300 ppm (18.4 and 21.9 mg/kg bw/day in males and females, respectively)</u>
Mean body weight parameters were unaffected by treatment.

Clinical chemistry determinations showed lower mean total bilirubin concentrations in females compared to controls. However, lower total bilirubin concentrations are considered not to be adverse effects of the test item, as they do not represent any functional impairment in the test organism.

Urinalysis revealed some cellular casts in a single male. Since this finding was also observed in one control male of a previous study conducted in the Wistar rat, it was considered not to be adverse.

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At necropsy, there was no treatment-related change in organ weight or macroscopic examinations in either sex. Microscopic examination showed treatment-related findings only in the kidney of males, where a slightly higher incidence and severity of hyaline droplets in proximal tubules was observed. As the change was also observed in some control males at the same severity, it was considered not to be adverse.

At 100 ppm (6.34 and 7.92 mg/kg bw/day in males and females, respectively)
The only treatment-related change in this group consisted of a decreased mean total bilirubin concentration in females only, compared to controls. As at the high and mid dose level, this change does not represent any functional impairment in the animal and is thus considered not to be adverse.

In conclusion, the dietary concentration of 300 ppm BCS-CN88460 (equating to 18.4 and 21.9 mg/kg bw/day in males and females, respectively) administered to male and female Wistar rat for at least 90 days was considered to be a No Observed Adverse Effect Level (NOAEL) in both sexes. This conclusion was based on the lower total bilirubin concentration noted in females and the limited changes noted at urinalysis and microscopic examination in the kidney of males, which were considered not to be adverse effects of treatment.

The hyaline droplet nephropathy noted in the kidney of males at 1000 and 300 ppm is a recognized lesion of male rats which is related to the accumulation of alpha2u-globulin the proximal tubules of affected animals. This protein is naturally reabsorbed and accumulated in the renal tubular epithelium of the young male rat. The sequence of renal events leading to its additional accumulation is dependent on the interaction between a chemical substance and the alpha2u-globulin, and is specific to the male rat. As humans secrete only trace amounts of alpha2u-globulin, this mechanism is generally accepted as being not relevant to humans. Therefore, although the hyaline droplets observed here in the kidney of male rats were considered to be treatment-related, they are considered not to be toxicologically relevant to man.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	White solid
Lot / Batch #:	NLL 8674-21-4
Purity:	97.7%
CAS #	1255734-28-1
Stability of test compound:	Until 26 January 2013, re-certified until 25 July 2013

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	rat
Strain:	Wistar Rj:WI (IOPS HAN)
Age:	6-7 weeks
Weight at dosing:	197-230g (males), 158-200g (females)
Source:	[redacted]
Acclimation period:	13 days
Diet:	A04CP1-10 diet, ad libitum
Water:	Filtered and softened tap water from the municipal water supply, ad libitum
Housing:	By sex in groups of 5, suspended stainless steel and wire mesh cages

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Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 per hour
Photoperiod:	12 hours dark, 12 hours light

B. STUDY DESIGN

1. **In life dates:** 24 October 2012 – 8 March 2013

2. **Animal assignment and treatment**
During the acclimatization phase, all animals were weighed, checked daily for moribundity and mortality, and subjected to a physical and ophthalmological examination. The day before the beginning of test item administration, all animals were weighed. A computerized randomization procedure (Pristima, version 6.3.2 build 17, Xybion Corp.) that ensured a similar body weight distribution among groups for each sex was used to select animals for the study from the middle of the weight range of available animals. Selected animals were in a weight range from 197 to 230g for the males, and from 158 to 200g for the females at the start of dosing.

3. **Dose selection**
The doses for this study were set on the basis of the 28-day study summarized in Point 5.3.1/01 of this document. In that study, male and female rats were administered BCS-CN88460 via the diet at 0, 300, 1000, and 3000 ppm.

At 3000 ppm, there were slight changes in some hematological and clinical chemistry parameters. Total cholesterol was increased in males, while total bilirubin was decreased in both males and females. Liver and thyroid weight were increased in both sexes, at gross necropsy observations in the liver included enlarged or dark liver, or prominent liver lobulation, and at microscopic examination treatment-related findings were observed in the liver, kidney, and thyroid gland in males and in the liver and thyroid gland in females.

At 1000 ppm in the 28-day study, total bilirubin was decreased in both males and females, while liver weight was increased and gross pathology showed enlarged or dark liver in both sexes. At microscopy, treatment-related findings were noted in the kidney in males and in the liver in females.

At 300 ppm in the 28-day study, total bilirubin was decreased in both males and females, and treatment-related findings were noted in the kidney in males only.

It was anticipated that the alpha2u-globulin accumulation in the kidney of the male rats would worsen significantly during the longer dosing period of the 90-day study, and that the CAR-PXR-mediated effects observed in the liver and thyroid in the 28-day study would be observed at a similar or greater magnitude in the 90-day study.

3. **Diet preparation and analysis**
The test item was incorporated into the diet by dry mixing to provide the required dietary concentrations of 100, 300, and 1000 ppm. There was one preparation for each concentration for the study. When not in use, the diet formulations were stored at room temperature.

The stability of the test item in the diet has been demonstrated in a previous study at concentrations of 60 and 10000 ppm over a 100-day storage period at room temperature. The homogeneity of BCS-CN88460 in the diet was verified on the first load of the

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formulations at the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean values obtained from the homogeneity check were taken as measured concentrations. The dietary levels of the test item were verified for each concentration on each preparation.

The homogeneity analysis showed that the prepared diets were at 94-98% of nominal concentration, while concentration analysis was at 95-100% of the nominal concentration.

4. Statistics

Statistical analyses were carried out using Prostima, version 6.3.2 build 17, Xybion Corp. Means and standard deviations were calculated for each group. All statistical analyses were carried out separately for males and females. Group means were compared at least at the 0.1% levels of significance.

In the main study, for body weight change parameters, terminal body weight, absolute and relative organ weight, selected hematology and clinical chemistry parameters, quantitative urinalysis parameters, spontaneous motor activity, fore- and hind-limb grip strength, landing foot splay, and rectal temperature parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

In the main study, for body weight parameters and selected hematology parameters, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation for body weight parameters or the square root transformation for hematology parameters. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on log-transformed data. If the ANOVA on log-transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on log-transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

In the main study, for urine pH, the group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the

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Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

In the reversibility phase, for body weight change, terminal body weight, absolute and relative organ weight, selected hematology and clinical chemistry parameters, and quantitative urinalysis parameters, the mean and standard deviation were calculated for each group, and per time period for body weight change parameters. Values were first compared using the F test; if the F test was significant ($p \leq 0.05$), a 2-sided modified t-test was used. If the F test was not significant ($p > 0.05$), a 2-sided T-test was used.

In the reversibility phase, for body weight and selected hematology parameters, the mean and standard deviation were calculated for each group. Values were first compared using the F test; if the F test was not significant ($p > 0.05$), a 2-sided T-test was carried out. If the F test was significant ($p \leq 0.05$), the data were transformed using the log transformation for body weight parameters or the square root transformation for hematology parameters, and an F test was applied to the transformed data. If the F test using transformed data were significant ($p \leq 0.05$), a 2-sided modified t-test was applied, while if the F test on transformed data were not significant ($p > 0.05$), a 2-sided T test was applied to the transformed data.

For urine pH measured in the reversibility phase, group means were compared using the non-parametric Mann-Whitney test (2-sided). If one or more group variances were equal to 0, means were compared using the non-parametric Mann-Whitney test (2-sided).

C. METHODS

1. Observations

Animals were checked for moribundity and mortality twice daily, once daily on weekends and public holidays, and were observed for clinical signs at least once daily throughout the study. Detailed physical examinations were performed at least weekly during the treatment and recovery periods. The nature, onset, severity, duration, and recovery of clinical signs were recorded.

2. Body weight

Each animal was weighed three times during the acclimatization period, on the first day of test item administration, then at least weekly throughout the treatment and recovery periods. Additionally, diet-fasted animals were weighed before scheduled necropsy to determine terminal body weight.

3. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded twice weekly during the first six weeks of treatment, then weekly for all animals during the treatment and recovery period. The mean weekly achieved compound intake, and intake for the entire study period, was calculated for each sex on the basis of body weight and food consumption.

4. Ophthalmoscopic examination

During the acclimatization period, all animals were subjected to an ophthalmic examination. After instillation of an atropinic agent, each eye was examined by means of an indirect ophthalmoscope. During week 13 of the treatment period, all surviving animals from the control and high dose groups were re-examined.

5. Neurological examination

During study week 11, a neurotoxicity assessment was performed for all surviving animals. Each animal was individually tested, with the order of animal testing randomly determined,

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and the observer was unaware regarding the animal's group assignment. The neurological examinations consisted of a Functional Observation Battery and a determination of exploratory motor activity.

Functional Observation Battery

- Home cage observation – while the animal was in its home cage, observations were collected regarding posture, piloerection, involuntary motor movements, gait abnormalities, vocalizations, or any abnormal behavior.
- Observation during handling – including ease to remove from cage, reaction to being handled, muscle tone, eyelid, lacrimation, salivation, nasal discharges, staining, or any other signs such as alopecia, emaciation, temperature upon touching.
- Open-field observation – each animal was individually observed in an open field for 2 minutes for piloerection, respiration, arousal, gait abnormalities, posture, involuntary motor movements, stereotypic movements, vocalizations, number of rearings, urine and feces spots.
- Reflex and physiological observations or measurements, including –
  - 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 and rapidity to regain 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 and strength of the flexor response of each hindlimb
  - Auditory startle response, by evaluating the animal's response to an auditory stimulus
  - Tail pinch response, by pinching the tail with a forceps and evaluating the animal's reaction
  - Grip strength, measured quantitatively for both fore- and hindlimbs of each animal using a grip strength apparatus equipped with one pull and one push strain gauge
  - Landing foot splay, where 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 motor activity

Animals were tested individually using an automated photocell recording apparatus designed to measure quantitatively spontaneous exploratory motor activity in a novel environment. Spontaneous motor activity was recorded during the first 60 minutes, with data collected at regular intervals throughout the session.

6. Hematology and clinical chemistry

On the day of scheduled sacrifice, blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus prior to sacrifice. An approximately equal number of animals, randomly distributed among all groups, were sampled on each day. Animals were diet-fasted overnight prior to bleeding, and were anesthetized by inhalation of isoflurane. Blood was collected on EDTA for hematology, on clot activator for serum clinical chemistry, and on sodium citrate for coagulation parameters. Blood smears were prepared and stained using May-Gruenwald-Giemsa method, and were saved for examination of the results of the hematology measurements were abnormal. Any significant

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change in the general appearance of the serum was recorded during clinical chemistry examinations.

7. Urinalysis

On study days 87 or 88 for main study animals, and recovery day 28 for animals in the reversibility phase, overnight urine samples were collected from all animals in all groups. An approximately equal number of animals randomly distributed among all groups were sampled on days 87 and 88. Food and water were not accessible during urine collection. Any significant change in the general appearance of the urine was recorded. Urine samples were weighed to determine urinary volume, pH was assayed, and urinary refractive index was measured. Semi-quantitative parameters were determined, and the microscopic examination of the urinary sediment was performed after centrifugation of the urine.

8. Sacrifice and pathology

On study days 93, 94, or 95 of the dosing phase, or day 30 or 31 of the recovery phase, all animals from all groups were sacrificed by exsanguination under deep anesthesia achieved through inhalation of isoflurane. An approximately equal number of animals randomly distributed among all groups were sampled on each day at the dosing phase and recovery sacrifices. Animals were diet-fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices, and all major organs, tissues and body cavities. Macroscopic abnormalities were recorded and sampled.

Table 5.3.2/01-1: Organs and tissues which were sampled and / or weighed at necropsy

	Digestive system		**Cardiovasc. / Hemat.**		**Neurologic**
X	Tongue	X	Aorta*	XX	Brain*+
X	Submaxillary (salivary) gland*	XX	Heart*+	X	Sciatic nerve*
		X	Bone marrow, sternum*	X	Spinal cord (cervical, thoracic, lumbar)*
X	Esophagus*	X	Lymph node, mesenteric*	X	Eyes*
X	Stomach*			X	Optic nerves*
X	Duodenum*	X	Lymph node, submaxillary*	XX	Pituitary gland*
X	Jejunum*				
X	Ileum*	XX	Spleen*+		**Glandular**
X	Cecum*	XX	Thymus*+	XX	Adrenal gland*+
X	Colon*		**Urogenital**	^	Lachrymal exorbital gland
X	Rectum*	XX	Kidney*+		
XX	Liver*+	X	Urinary bladder*	X	Parathyroid gland*
X	Pancreas	XX	Testis*+	XX	Thyroid gland*
	Respiratory	XX	Epididymis*+	X	Harderian gland
X	Trachea*	XX	Prostate gland*		
X	Lung*	X	Seminal vesicle*		**Other**
^	Nasal cavities*	XX	Ovary*+	X	Bone (sternum)
^	Pharynx*	XX	Uterus (with cervix)*+	X	Skeletal muscle
^	Larynx*	X	Mammary gland*	X	Skin*
		X	Vagina	X	All gross lesions and masses
				X	Articular surface (femorotibial joint)

* recommended for 90-day oral rodent studies based on US EPA guideline 870.3100
organ weights required for rodent studies
X tissues were collected for histological examinations
XX organs were weighed fresh at scheduled sacrifice only, with paired organs weighed together, and collected for histological examination
^ organs were preserved for possible micropathologic evaluation

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II. RESULTS AND DISCUSSION

A. OBSERVATIONS AND MORTALITY

1. Clinical signs of toxicity

No treatment-related clinical signs were noted at any dose level in either males or females.

2. Mortality

There were no mortalities in either sex.

3. Neurological examinations

There were no treatment-related effects noted in the FOB observations in either males or females with regard to behavior in the home cage, during handling, or in the open field, or in reflexes or responses evaluated. No treatment-related changes were noted in grip strength, landing foot splay or rectal temperature in either males or females. Finally, no treatment-related changes were observed in motor activity parameters in either sex at any dose.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 1000 ppm in males, mean overall body weight gain was statistically non-significantly reduced by 12% relative to controls, while mean body weight was statistically non-significantly reduced by 7% compared to controls. In females at 1000 ppm, mean overall body weight gain was decreased relative to controls by 8%, although this was not statistically significant. Mean body weight in females at 1000 ppm was similar to control values. Both body weight and body weight gain at 100 and 300 ppm were similar to controls.

In the recovery group males at 1000 ppm, mean body weight was slightly lower than the control group throughout the 4-week recovery phase. Body weight gain during the first week of the recovery phase was increased relative to controls, although during the second and third week of recovery the increase over control body weight gain was less. At the end of the recovery phase, overall mean body weight gain was statistically non-significantly increased relative to controls. In 1000 ppm females of the recovery group, mean body weight and mean body weight gain were similar to those seen in controls. Although there is a statistically significant increase in body weight at the end of the recovery period, this is due to body weight loss in 5 of the 10 females in the control group, considered due to a problem with the water distribution system.

Based on this data, body weight and body weight gain were not markedly affected by dietary administration of BCS-CN88460 in male and female rats over a 90-day period.

Table 5.3.2/01-2: Mean body weight and body weight gain in male and female rats administered BCS-CN88460 in the diet for 90 days

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Main study phase	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Initial body wt, g	213.3	214.6	214.4	213.4	177.3	183.4	185.0	185.3*
Body wt, g, week 13	547.9	545.3	545.3	507.1	297.3	294.4	297.6	295.5
Body wt gain, g	334.6	330.7	330.9	293.6	120.0	111.0	112.6	110.1
Recovery phase								
Initial body wt, g	552.0			498.8	289.7			304.0
Body wt, g, day 29	573.1			536.5	285.0			310.4*
Body wt gain, g	21.1			37.7	- 4.7			6.4

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

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C. FOOD CONSUMPTION AND COMPOUND INTAKE

Food consumption was not affected in either males or females in either the main study phase or the recovery phase.

The mean achieved dose levels of BCS-CN88460 in this study are shown in Table 5.3.2/01-3.

Table 5.3.2/01-3: Mean achieved dose levels of BCS-CN88460, in mg/kg bw/day, in male and female rats administered the compound via the diet

	BCS-CN88460, dietary concentration in ppm					
	Males			Females		
	100	300	1000	100	300	1000
Weeks 1-13	6.34	18.4	63.5	7.92	21.9	80.9

D. OPHTHALMOSCOPIC EXAMINATION

There were no treatment-related abnormalities at ophthalmological examination at the end of the study.

E. HEMATOLOGY, CLINICAL CHEMISTRY, AND URINALYSIS

1. Hematology

No treatment-related changes in hematological parameters were noted in either males and females of either the treatment or the recovery phases.

2. Clinical Chemistry

In the treatment phase animals, total bilirubin was statistically decreased in males at 1000 ppm only. In females, there was a dose-related decrease in total bilirubin at all doses, which was statistically significant at 300 and 1000 ppm. Total cholesterol was also slightly but statistically significantly increased in females at 1000 ppm.

After 4 weeks of recovery phase, neither total bilirubin nor total cholesterol were different from control animals in a biologically meaningful way; total cholesterol was decreased in the 1000 ppm recovery males and females, however a decrease in total cholesterol is not toxicologically significant.

Table 5.3.2/01-4: Mean total bilirubin and total cholesterol in male and female rats administered BCS-CN88460 via the diet for 13 weeks

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Main study phase	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Total bilirubin, µmol/L	0.94	0.97	0.96	0.56*	1.85	1.52	1.16*	0.68**
Total cholesterol, mmol/L	1.528	1.639	1.750	1.716	1.933	1.838	1.973	2.302*
Recovery phase								
Total bilirubin, µmol/L	1.13			0.84	1.70			1.79
Total cholesterol, mmol/L	1.584			1.403*	1.889			1.638*

Significant at * $p \leq 0.05$, ** $p \leq 0.01$; *** $p \leq 0.001$.

3. Urinalysis

No urinalysis changes were noted in females at any dose. In males at 1000 ppm, there were cellular casts in the urine of 5 of 10 animals. This finding was considered to be treatment-related as it was associated in these animals with microscopic changes in the kidney. Although one animal at 300 ppm also had cellular casts in the urine, this was not considered to be a treatment-related finding, as in a previous study one control male had been noted with cellular casts in the urine.

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In the recovery phase males, no cellular casts were observed in urine of males at 1000 ppm, indicating that this finding was related to dietary administration of BCS-CN88460.

F. SACRIFICE AND PATHOLOGY

1. Terminal body weight and organ weight

In the main study, at 1000 ppm, mean terminal body weight was statistically non-significantly decreased relative to controls. Treatment-related organ weight changes were limited to increased absolute and relative liver weights in females at 1000 ppm and increased relative thyroid weight in males at 1000 ppm.

In the reversibility group, in males at 1000 ppm, mean terminal body weight was statistically non-significantly decreased relative to controls. Although mean relative liver weight was increased at 1000 ppm, this is considered to be due to the decreased body weight, as there was no change in absolute liver weight between control and treated animals. This is supported by the absence of an effect on liver weight relative to brain weight in the 1000 ppm males. Terminal body weight and the absolute and relative weights of the liver were similar to controls in the 1000 ppm females.

Thus, the increased absolute and / or relative weights of liver in the females and of thyroid in the males, as observed at the end of the treatment phase, were considered to be treatment-related and reversible.

Table 5.3.2/01-5: Mean terminal body weight and absolute and relative weights of liver and thyroid in male and female rats administered BCS-CN88460 via the diet for 13 weeks

	BCS-CN88460, dietary concentration in ppm			
Main study phase	**0**	**100**	**300**	**1000**
Males				
Terminal body wt, g	520.7	517.9	515.5	486.2
Liver wt, g	11.24	11.27	11.17	11.67
Liver wt, % body wt	2.162	2.175	2.163	2.397**
Liver wt, % brain wt	508.659	520.495	505.156	537.217
Thyroid gland, g	0.0188	0.0200	0.0179	0.0213
Thyroid wt, % body wt	0.00363	0.00386	0.00347	0.00439*
Thyroid wt, % brain wt	0.84952	0.92602	0.81330	0.98505
Recovery phase				
Terminal body wt, g	554.8			509.6
Liver wt, g	10.75			10.67
Liver wt, % body wt	1.940			2.093*
Liver wt, % brain wt	478.590			492.287
Thyroid gland, g	0.0210			0.0202
Thyroid wt, % body wt	0.00381			0.00403
Thyroid wt, % brain wt	0.93985			0.93031
Females				
Terminal body wt, g	287.6	277.1	284.0	272.1
Liver wt, g	6.207	6.240	6.342	7.469**
Liver wt, % body wt	2.158	2.254	2.231	2.754***
Liver wt, % brain wt	303.846	303.305	311.340	358.686**
Thyroid gland, g	0.0153	0.0144	0.0159	0.0150
Thyroid wt, % body wt	0.00533	0.00520	0.00558	0.00553
Thyroid wt, % brain wt	0.74541	0.69838	0.77840	0.72228

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Table 5.3.2/01-5 continued

	BCS-CN88460, dietary concentration in ppm			
Main study phase	**0**	**100**	**300**	**1000**
Recovery phase				
Terminal body wt, g	283.3			292.4
Liver wt, g	5.87			6.41
Liver wt, % body wt	2.066			2.195
Liver wt, % brain wt	281.325			305.455
Thyroid gland, g	0.0156			0.0154
Thyroid wt, % body wt	0.0051			0.00531
Thyroid wt, % brain wt	0.75012			0.73405

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

2. Gross pathology

At 1000 ppm, enlarged liver was noted in 8/10 females and 2/10 males. In the females, this corresponded with microscopic findings and is thus considered to be treatment related. One female at 100 and one female at 300 ppm were noted to have enlarged liver, but there were no histopathological correlates.

In the recovery phase, one male and two females at 1000 ppm had enlarged liver. However, there were no findings at microscopic examination and thus this finding is considered not to be related to treatment.

3. Microscopic pathology

In the liver, minimal to slight periportal to panlobular hepatocellular hypertrophy was noted in main-study females at 1000 ppm. There were no findings in the liver of reversibility-phase animals.

Table 5.3.2/01-6: Incidence of treatment-related findings in the liver of male and female rats administered BCS-CN88460 via the diet for 13 weeks

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Main study phase	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
N examined	10	10	10	10	10	10	10	10
Hepatocellular hypertrophy: periportal to panlobular								
Minimal	0	0	0	0	0	0	0	5
Slight	0	0	0	0	0	0	0	1
Total	0	0	0	0	0	0	0	6

At 1000 ppm, in males of the main study, there was an increase in the incidence and severity of hyaline droplets in the proximal tubules, which was associated with a slightly higher severity of bilateral basophilic tubules. Some males at 1000 ppm also had minimal granular cast(s). At 300 ppm in males, there was a slightly higher incidence of hyaline droplets in the proximal tubules. This finding was considered to be treatment-related but not adverse, as some control males also had hyaline droplets in the proximal tubules. In the recovery phase males at 1000 ppm, there was still an increase in the incidence and severity of bilateral basophilic tubules, as well as the presence of granular cast(s).

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Table 5.3.2/01-7: Incidence of treatment-related findings in the kidney of male and female rats administered BCS-CN88460 via the diet for 13 weeks

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Main study phase	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
N examined	10	10	10	10	10	10	10	10
Hyaline droplets: proximal tubules								
Minimal	3	4	5	4	0	0	0	0
Slight	1	0	2	5	0	0	0	0
Moderate	0	0	0	1	0	0	0	0
Total	4	4	7	10	0	0	0	0
Basophilic tubules: bilateral								
Minimal	4	0	0	1	0	0	0	0
Slight	0	0	1	5	0	0	0	0
Total	4	0	1	6	0	0	0	0
Granular cast(s)								
Minimal	0	0	0	2	0	0	0	0
Total	0	0	0	2	0	0	0	0
Recovery phase								
N examined	10			10	10			10
Basophilic tubules: bilateral								
Minimal	2			4	0			1
Slight	0			1	0			0
Total	2			5	0			1
Granular cast(s)								
Minimal	0			3	0			0
Total	0			3	0			0

In the thyroid, both males and females of the main study at 1000 ppm had minimal follicular cell hypertrophy and colloid alteration. In the reversibility group, the incidence, but not the severity, of colloid alteration was still increased at 1000 ppm in males.

Table 5.3.2/01-8: Incidence of histopathological findings in the thyroid in male and female rats administered BCS-CN88460 via the diet for 13 weeks

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Main study phase	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
N examined	10	10	10	10	10	10	10	10
Follicular cell hypertrophy: diffuse								
Minimal	1	0	1	5	0	0	0	5
Total	1	0	1	5	0	0	0	5
Colloid alteration								
Minimal	2	0	1	5	0	0	0	2
Total	2	0	1	5	0	0	0	2
Recovery phase								
N examined	10			10	10			10
Colloid alteration								
Minimal	2			6	0			1
Slight	1			0	0			0
Total	3			6	0			1

4. Quantification of alpha2u-globulin

In control animals, small alpha2u-globulin-positive droplets were observed in all males with a minimal to slight severity. The severity of the observation of droplets staining positive for alpha2u-globulin increased in a dose-related manner at all doses. Additionally, the size and

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shape of the droplets were changed at 1000 ppm (moderately sized to large polyangular), and the number of affected tubules increased at 1000 ppm as well (up to 50 75%).

This clearly demonstrates the nature of the hyaline droplets observed in the kidney. A dose-related accumulation of alpha2u-globulin in the renal proximal tubules was observed, and was correlated with the higher incidence and severity of hyaline droplets in proximal tubules. These findings were shown to be reversible, with no appreciable difference after four weeks between the control and the 1000 ppm animals. Thus, dietary administration of BCS-CN88460 increases the male rat-specific accumulation of alpha2u-globulin in the kidney tubule.

Table 5.3.2/01-9: Incidence and mean severity of alpha2u-globulin accumulation in the kidney of male rats administered BCS-CN88460 via the diet for 13 weeks

	BCS-CN88460, dietary concentration in ppm			
Main phase	**0**	**100**	**300**	**1000**
N examined	10	10	10	10
Alpha2u-globulin immunohistochemical staining				
Minimal	6	7	5	2
Slight	4	3	4	6
Moderate	0	0	1	2
Total	10	10	10	10
Mean severity	1.40	1.30	1.60	2.00
Recovery phase				
N examined	9			10
Alpha2u-globulin immunohistochemical staining				
Minimal	7			7
Slight	2			3
Total	9			10
Mean severity	1.22			1.30

G. DEFICIENCIES

No specific deficiencies are noted. Toxicokinetic data were not collected because at the time the study was conducted this was not a requirement nor was it a part of the standard study protocol. As summarized in the summary of the short-term toxicity section above, the toxicokinetic data obtained in the rat developmental toxicity study and at the 90-day timepoint of the chronic study can be used as a general indication of the behavior of BCS-CN88460 after oral administration.

III. CONCLUSIONS

Based on the changes observed in male rats at 1000 ppm in urinalysis and microscopic examination of the kidneys, the No Observed Adverse Effect Level for this study with BCS-CN88460 is 300 ppm (18.4 mg/kg bw/day in males, 21.9 mg/kg bw/day in females).

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Report:	KCA 5.3.2/02; [redacted]; 2013; M-472773-01-1
Title:	BCS-CN88460 - 90-day toxicity study in the mouse by dietary administration
Report No.:	M-472773-01-1
Document No.:	M-472773-01-1
Guideline(s):	OECD guideline 408 (September, 1998) EEC Directive 2001/59/EC, Method B.26 (August, 2001) US EPA OCSPP guideline number 870.3100 MAFF in Japan 12 Nousan n°8147 (November, 2000)
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Executive Summary
BCS-CN8460 was administered continuously via the diet to groups of C57BL/6J mice (10 per sex per dose group) at concentrations of 0, 100, 300, and 1000 ppm (equivalent to 0, 17.0, 51.0, and 168 mg/kg bw/day in males and 0, 19.5, 59.8, and 207 mg/kg bw/day in females, respectively) for at least 90 days. Animals were observed daily for mortality and clinical signs. Body weight and food consumption were measured weekly. A detailed physical examination was performed weekly throughout the study. On the day of necropsy, a blood sample was collected from the retro-orbital venous plexus of all animals for selected clinical chemistry determinations. All animals were necropsied, selected organs were weighed, and designated tissues were taken, fixed, and examined microscopically.

Up to the highest dietary concentration tested (1000 ppm), dietary administration of BCS-CN88460 to C57BL/6J mice induced no mortalities, no treatment-related clinical signs, and no treatment-related effects on body weight or food consumption parameters.

At 1000 ppm (168 and 207 mg/kg bw/day in males and females, respectively)
Clinical chemistry evaluation showed lower total bilirubin concentrations in both sexes. At necropsy, mean absolute and relative liver weights were 12-18% higher in both sexes when compared to controls, which was considered to be an adverse effect. At microscopic examination, a higher incidence of diffuse mainly centrilobular hepatocellular vacuolation was observed concomitantly with a lower incidence of diffuse hepatocellular vacuolation when compared to controls. These changes corresponded to a loss of vacuolation in the periportal area of the hepatic lobule.

At 300 ppm (51.0 and 59.8 mg/kg bw/day in males and females, respectively)
Clinical chemistry evaluation showed lower total bilirubin concentrations in both sexes. At necropsy, mean absolute and relative liver weights were 8% higher in males when compared to controls. At microscopic examination, a higher incidence of diffuse mainly centrilobular hepatocellular vacuolation was observed in females only, concomitantly with a lower incidence of diffuse hepatocellular vacuolation when compared to controls.

As they do not represent any functional impairment in the animal and in absence of hepatocellular degeneration or necrosis in the liver, all these changes were considered not to be adverse.

At 100 ppm (17.0 and 19.5 mg/kg bw/day in males and females, respectively)
The only treatment-related effect observed consisted of a tendency towards lower total bilirubin concentrations in both sexes, without any accompanying changes observed in organ weights or at histopathological examination.

As they do not represent any functional impairment in the animal and in the absence of hepatocellular degeneration or necrosis in the liver, all these changes were considered not to be adverse.

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In conclusion, a dose level of 300 ppm (equivalent to 51.0 and 59.8 mg/kg bw/day in males and females, respectively) was considered to be a No Observed Adverse Effect Level (NOAEL) in the male and female C57BL/6J mice, based on the minor changes noted at this dietary concentration which do not represent an alteration in the general function of the animal.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	White solid
Lot / Batch #:	NLL 8674-214
Purity:	97.7%
CAS #	1255734-28-1
Stability of test compound:	Until 26 July 2012 / 25 Jun 2013 (re-certification)

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	mouse
Strain:	C57BL/6J
Age:	6-7 weeks old
Weight at dosing:	18.7-22.2g, males; 15.5-19.0g, females
Source:	[redacted]
Acclimation period:	6 days
Diet:	A04CP1-10 from [redacted]
Water:	Filtered and softened tap water from the municipal water supply
Housing:	Individually in suspended, stainless steel and wire mesh cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 per hour
Photoperiod:	12 hours dark, 12 hours light

B. STUDY DESIGN

1. In life dates: 16 October 2012-18 January 2013

2. Animal assignment and treatment

All animals were weighed at least weekly, checked at least daily for moribundity and mortality, and subjected to a physical examination once during the acclimatization phase. The day before the first test item administration, all animals were weighed and randomized, and a computer randomization procedure (Pristima, version 6.3.2, build 17, Xybion Corp) that ensured a similar body weight distribution among groups for each sex was used to select animals for the study from the middle of the weight range of the available animals.

The dose levels for this study were set on the basis of the results observed in the 28-day mouse study (see data point 5.3.1//02), in which animals were administered BCS-CN88460 via the diet at concentrations of 0, 200, 800, or 2000 ppm. In that study, the liver was the only target organ in either males or females. At 200 ppm, the only treatment-related change observed was a decrease in total bilirubin in females. At 800 ppm, aspartate

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aminotransferase activities were increased in males, total bilirubin was decreased in females, liver weight was increased by 10-14% in females, and the incidence of necrotic foci and focal single cell necrosis in the liver was increased in males. At 2000 ppm, the findings observed included increased activities of aspartate and alanine aminotransferase and alkaline phosphatase, increased liver weight, and histopathological findings including centrilobular hypertrophy, necrotic foci, and focal single cell necrosis. Thus, the dose levels for the current, 90-day dietary study in the mouse were set at 0, 100, 300, and 1000 ppm.

3. Diet preparation and analysis

The test item was incorporated into the diet by dry-mixing to provide the required dietary concentrations of 100, 300, or 1000 ppm, with only one preparation made for the duration of the study. When not in use, the diet formulations were stored at room temperature.

The homogeneity of BCS-CN88460 in diet was verified at the lowest and highest concentrations, to demonstrate adequate formulation procedures. The mean values obtained from the homogeneity check were taken as measured concentrations. In addition, the dietary concentration of the test item was verified at the mid-dose concentration.

The homogeneity analysis demonstrated that the concentration was at 91-97% of the nominal concentration, while concentration analysis yielded 92-96% of the nominal concentration. These results were considered to be acceptable for use on the current study.

4. Statistics

Statistical analyses were carried out using Pristima, version 6.3.2 build 17, Xybion Corp. Means and standard deviations were calculated for each group. All statistical analyses were carried out separately for males and females. Group means were compared at least at the 0.1% levels of significance.

For body weight change parameters, terminal body weight, absolute and relative organ weight, and clinical chemistry parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight and food consumption parameters, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on log-transformed data. If the ANOVA on log-transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using

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the two-sided Dunnett test on log-transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

If one or more group variances were equal to 0, means were compared using non-parametric procedures.

C. METHODS

1. Observations

Animals were checked for moribundity and mortality twice daily, or once daily on weekends and public holidays, and were observed cage-side for clinical signs at least once daily. Detailed physical examinations were performed at least weekly during the treatment period.

2. Body weight

Each animal was weight on the first day of dietary test item administration, then at least weekly throughout the treatment period. Additionally, diet-fasted animals were weighed before scheduled necropsy to collect terminal body weight.

3. Food consumption and compound intake

The weight of food supplied, and of that remaining at the end of the food consumption period, was recorded weekly for all animals during the treatment period, and food spillage was also noted. From these records the mean daily consumption was calculated.

4. Ophthalmology

Ophthalmological examinations were not conducted during the study.

5. Clinical chemistry

Animals were diet fasted overnight before scheduled sacrifice, and on the day of scheduled sacrifice blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus prior to sacrifice. Animals were anesthetized by isoflurane inhalation, and blood was collected on clot activator for serum clinical chemistry.

6. Urinalysis

Urinalysis was not conducted in this study.

7. Sacrifice and pathology

On study days 92, 93, or 94, an approximately equal number of animals randomly distributed among all groups were diet-fasted overnight prior to sacrifice on the following day by exsanguination under deep anesthesia. Necropsy included examination of the external surfaces, all orifices, and all major organs, tissues, and body cavities. Macroscopic abnormalities were recorded, sampled, and examined microscopically.

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Table 5.3.2/02-1: Organs and tissues which were sampled and / or weighed at necropsy

	Digestive system		**Cardiovasc. / Hemat.**		**Neurologic**
X	Tongue	X	Aorta*	XX	Brain*+
X	Submaxillary (salivary) gland*	XX	Heart*+	X	Sciatic nerve*
		X	Bone marrow, sternum*	X	Spinal cord (cervical, thoracic, lumbar)*
X	Esophagus*	X	Lymph node, mesenteric*		
X	Stomach*			X	Eyes*
X	Duodenum*	X	Lymph node, submaxillary*	X	Optic nerves*
X	Jejunum*			XX	Pituitary gland*
X	Ileum*	XX	Spleen*+		
X	Cecum*	XX	Thymus*+		**Glandular**
X	Colon*			XX	Adrenal gland*+
X	Rectum*		**Urogenital**	^	Lacrymal exorbital gland
XX	Liver*+	XX	Kidney*+		
X	Pancreas	X	Urinary bladder*	X	Parathyroid gland*
		XX	Testis*+	XX	Thyroid gland*
	Respiratory	XX	Epididymis*	X	Harderian gland
X	Trachea*	XX	Prostate gland*		
X	Lung*	X	Seminal vesicle*		**Other**
^	Nasal cavities*	XX	Ovary*	X	Bone (sternum)
^	Pharynx*	XX	Uterus (with cervix)*+	X	Skeletal muscle
^	Larynx*	X	Mammary gland*	X	Skin*
		X	Vagina*	X	All gross lesions and masses
				X	Articular surface (femorotibial joint)

* recommended for 90-day oral rodent studies based on US EPA guideline 870.3100
\+ organ weights required for rodent studies
X tissues were collected for histological examinations
XX organs were weighed fresh at scheduled sacrifice only, with paired organs weighed together, and collected for histological examination
^ organs were preserved for possible micropathologic evaluation

For sacrificed animals, a femoral bone marrow smear was prepared and stained with May-Gruenwald Giemsa, but not examined as no treatment-related changes were observed in bone marrow histology.

Samples were fixed by immersion in 10% neutral buffered formalin with the exception of the eye, optic nerve, Harderian gland, epididymis, and testis, which were fixed in Davidson's fixative. Histological sections were prepared and stained with hematoxylin and eosin from all organs and tissue samples from all animals in the control and high dose groups, and from liver, lung, kidney, thyroid gland, and significant gross findings observed at necropsy for all animals in the intermediate dose group.

H. RESULTS AND DISCUSSION

A. CLINICAL SIGNS AND MORTALITY

1. Clinical signs of toxicity

There were no treatment-related clinical signs in either sex at any dose level.

2. Mortality

There were no mortalities during the study.

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B. BODY WEIGHT AND BODY WEIGHT GAIN

There were no treatment-related effects on mean body weight parameters in any dose group. Total body weight gain was reduced compared to controls in males at 100 ppm (86% of control body weight gain, not statistically significant) and 1000 ppm (81% of controls, $p \leq 0.05$), but in the absence of a relationship to dose it was considered to be not treatment related.

Table 5.3.2/02-1: Mean body weight and cumulative body weight gain in male and female mice administered BCS-CN88460 via the diet for 90 days

		BCS-CN88460, dietary concentration in ppm							
		Males				Females			
Parameter	**Day**	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Body weight, g	1	20.61	20.67	20.58	20.53	17.73	17.58	17.46	17.55
	29	21.83	21.69	21.86	21.62	20.76	20.52	20.45	20.28
	57	25.43	24.98	25.29	24.74	21.78	21.32	21.12	21.15
	92	26.44	25.69	26.42	25.25	22.40	21.92	21.70	22.10
Body weight gain, g	1-29	3.26	2.87	3.16	2.76	3.03	2.94	2.99	2.73
	1-57	4.82	4.31	4.71	4.21	4.05	3.74	3.66	3.60
	1-92	5.83	5.02	5.84	4.72*	4.67	4.34	4.24	4.55

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

C. FOOD CONSUMPTION AND COMPOUND INTAKE

There was no effect of treatment on food consumption in either males or females in any group.

The mean achieved dose levels of BCS-CN88460 in this study are shown in Table 5.3.2/02-2.

Table 5.3.2/02-2: Mean achieved dose levels of BCS-CN88460, in mg/kg bw/day, in male and female rats administered the compound via the diet

	BCS-CN88460, dietary concentration in ppm					
	Males			Females		
	100	**300**	**1000**	**100**	**300**	**1000**
Weeks 1-13	17.0	51.0	168	19.5	59.8	207

D. BLOOD ANALYSES

1. Clinical Chemistry

The only treatment-related change in any clinical chemistry parameter was a decrease in total bilirubin concentrations seen in both sexes at all dose levels. The decrease observed at the top dose of 1000 ppm was statistically significant and related to treatment in both males and females. The statistically significant decrease observed in females at 100 ppm is considered to be due to two low values induced by slight hemolysis.

In males at 1000 ppm, albumin and total cholesterol were statistically significantly decreased. However, the change in albumin concentration was very low in magnitude, and all individual values for total cholesterol were within the control range. Thus, these two observations were considered not to be related to treatment.

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Table 5.3.2/02-3: Mean values for total bilirubin, total cholesterol, and albumin in male and female mice administered BCS-CN88460 via the diet for 90 days

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	100	300	1000	0	100	300	1000
Total bilirubin, umol/L	1.23	0.88	0.88	0.71**	1.24	0.71**	0.92	0.84*
Total cholesterol, mmol/L	1.892	2.001	1.809	1.570*	1.564	1.547	1.576	1.557
Albumin, g/L	35.8	35.1	35.0	33.9**	35.6	35.9	35.8	35.7

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

E. SACRIFICE AND PATHOLOGY

1. Organ weight

Terminal body weight was not changed by dietary administration of BCS-CN88460. Absolute and / or relative liver weight were increased in males from 300 ppm and in females at 1000 ppm only. There were no other treatment-related effects on organ weight in either sex.

2. Gross and histopathology

There were no macroscopic findings which were considered to be related to treatment in either sex.

The only histopathological finding which was considered to be related to treatment was a shift in the pattern of hepatocellular vacuolation, with a loss of diffuse hepatocellular vacuolation and of vacuolation in the periportal area of the hepatic lobules, and a greater incidence of centrilobular hepatocellular vacuolation.

Table 5.3.2/02-3: Terminal body weight, mean absolute and relative liver weight, and treatment-related liver histopathological findings in male and female mice administered BCS-CN88460 via the diet for 90 days

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	100	300	1000	0	100	300	1000
Terminal body wt, g	22.19	21.69	22.26	20.99	18.86	18.11	17.98	18.21
Liver wt, g	0.912	0.918	0.989	1.018*	0.791	0.818	0.794	0.891*
Liver wt, % body wt	4.107	4.232	4.447*	4.840**	4.183	4.518*	4.410	4.886**
Liver wt, % brain wt	205.3	206.1	219.3	230.6	172.3	180.5	175.4	196.9
Hepatocellular vacuolation, mainly centrilobular, diffuse								
Minimal	1	0	0	3	1	0	3	4
Slight	0	0	0	0	1	1	1	4
Total	1	0	0	3	2	1	4	8
Hepatocellular vacuolation, diffuse								
Minimal	1	5	1	2	2	1	3	0
Slight	8	5	9	5	6	8	3	1
Total	9	10	10	7	8	9	6	1

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

F. DEFICIENCIES

No deficiencies were noted. Toxicokinetic data were not collected because at the time the study was conducted this was not a requirement nor was it a part of the standard study protocol. As summarized in the summary of the short-term toxicity section above, the toxicokinetic data

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obtained in the rat developmental toxicity study and at the 90-day timepoint of the chronic study can be used as a general indication of the behavior of BCS-CN88460 after oral administration.

III. CONCLUSIONS

Based on the increase in absolute and relative liver weight in both males and females (12-18%) and the accompanying shift in hepatocellular vacuolation to a pattern of centrilobular vacuolation, the NOAEL in both males and females was established at 300 ppm, approximately 51.0 mg/kg bw/day in males and 59.8 mg/kg bw/day in females.

Report:	KCA 5.3.2/03; [redacted] 2015; M-520001-01-1
Title:	BCS-CN88460: 90-Day toxicity study in the dog by dietary administration
Report No.:	SA 13272
Document No.:	M-520001-01-1
Guideline(s):	OECD guideline 409 (1998) EEC Directive 67/548/EEC, Annex V, Method B.27 (June, 1967) amended by Commission Directive 2001/59/EC, Method B.27 (August, 2001) US EPA OCSPP Guideline number 870.3150 MAFF in Japan, notification 12 Nousan n°8147 (November, 2000)
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Executive Summary

BCS-CN88460 was administered by dietary inclusion to groups of 4 male and 4 female beagle dogs at dietary concentrations of 0, 170, 500, or 1500 ppm (equating approximately to 0, 5.5, 15.9, and 50.4 mg/kg bw/day in males and 0, 5.5, 16.2, and 54.0 mg/kg bw/day in females) for at least 13 weeks. Each animal was checked for ill health, moribundity, and mortality twice daily or once daily on weekend and public holidays during the acclimatization phase and throughout the study. Food consumption was recorded daily throughout the study, and body weight was measured weekly as well as prior to necropsy. A detailed physical examination was performed prior to the start of treatment and every month throughout the treatment period. Ophthalmological examination was performed during the acclimatization phase and at the end of the study. Blood analysis (hematology and clinical chemistry) and urinalysis were performed once during the acclimatization phase, at week 7, and at the end of the study. A blood sample was also collected from selected animals for bioanalytical examination at the end of the study. All animals were subjected to a detailed necropsy. Selected organs were weighed, and a range of tissues were collected and processed for histopathological examination.

Up to the highest dietary concentration tested, 1500 ppm, dietary administration of BCS-CN88460 to beagle dogs for at least 13 weeks induced no mortalities, no treatment-related changes in food consumption, and no relevant changes at ophthalmological examination, hematology determinations, urinalysis parameters, or gross observation.

Near the end of the study (week 12), the bioanalytical examination showed that plasma concentrations of BCS-CN88460 before food distribution were marginally above the limit of quantification in the high dose group in both sexes, and were below or close to the limit of quantification in the mid and low dose groups in both sexes. After food distribution, the mean values in the treated groups showed a dose-related increase in plasma concentrations of the test item, with both sexes showing similar results at both the 4-hour and the 7-hour time points, and a peak concentration noted 4 hours after food distribution.

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At 1500 ppm (50.4 and 54.0 mg/kg bw/day in males and females, respectively)
The only treatment-related clinical signs consisted of increased salivation noted for ¾ females at 5 and 7 occasions during the second half of the study.

Mean body weight parameters were unaffected by treatment during the first half of the study, whereas twoards the end of the study mean body weight was 7% and 8% lower than controls in males and females, respectively. Overall mean body weight gain was 39% lower than controls in both sexes.

Clinical chemistry evaluation revealed higher mean alkaline phosphatase activity at week 7 and week 12-13 in males and females, together with decreased mean bilirubin concentrations at weeks 8 and 12-13 in males and females when compared to controls.

At necropsy, mean terminal body weight was decreased by 6% in males and 9% in females, and mean liver weight parameters were increased by 34-43% in males when compared to controls. At the microscopic examination, minimal centrilobular hepatocellular hypertrophy was observed in 3/4 males, together with cytoplasmic changes (eosinophilic intracytoplasmic vacuoles) in the liver for 2 of these males.

At 500 ppm (15.9 and 16.2 mg/kg bw/day in males and females, respectively)
The only treatment-related changes were noted at the clinical chemistry determination, where higher mean alkaline phosphatse activities were obesrved at weeks 7 and weeks 12-13 in males and females, together with lower mean bilirubin concentrations at weeks 7 and 12-13 in males and at weeks 12-13 in females when compared to controls. These changes were considered to be treatment-related, but not to be adverse effects as they either consist only of an adaptive response to the treatment (higher alkaline phosphatase activities) or do not represent any functional impairment in the animal (lower mean bilirubin concentrations).

At 170 ppm (5.5 mg/kg bw/day in both males and females
The only treatment-related changes were noted at the clinical chemistry evaluation at weeks 7 and 12-13, where higher mean alkaline phosphatase activities were observed in males and females, together with lower mean bilirubin concentrations in females when compared to controls. These changes were considered to be treatment-related, but not to be adverse effects, as they are either an adaptive response to the treatment (higher alkaline phosphatase activities) or do not represent any functional impairment in the animal (lower mean bilirubin concentrations).

In conclusion, a dietary concentration of 1500 ppm (equating to 50.4 and 54.0 mg/kg bw/day in males and females, respectively) administered to beagle dogs for at least 13 weeks induced significant toxicity, which essentially consisted of a 38% reduction in the overall mean body weight gain and a higher mean alkaline phosphatase activity in both sexes, together with a 34-43% increase in mean liver weight parameters and microscopic changes in the liver of males. A dose level of 500 ppm (equating to 15.9 and 16.2 mg/kg bw/day in males and females, respectively) was considered to be a No Observed Adverse Effect Level (NOAEL) in both sexes.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	White solid
Lot / Batch #:	2013-006492
Purity:	94.2%
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

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2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	dog
Strain:	beagle
Age:	8-9 months old
Weight at dosing:	5.9-8.6 kg, males; 6.1-7.3 kg, females
Source:	[redacted]
Acclimation period:	20 days
Diet:	[redacted], France
Water:	Filtered and softened tap water from the municipal water supply
Housing:	Individually in stainless steel kennels with a floor surface area of 2m2, during the day, where possible, they were pair-housed overnight by temporary opening of the partitions between 2 dogs from the same sex and dose group. Each week, supervised exercise was permitted in inside runs during cleaning of cages. During the exercise, dogs were separated by sex and dose group.
Environmental conditions:	
Temperature:	18-21°C
Humidity:	40-70%
Air changes:	Target of 15 per hour
Photoperiod:	12 hours dark, 12 hours light

B. STUDY DESIGN

1. In life dates: 12 February 2014-6 June 2014

2. Animal assignment and treatment

Shortly after arrival, all dogs were examined by a veterinarian for signs of ill health and were subjected to a detailed clinical examination to assess their physical and behavioral status. During the acclimatization phase, animals were checked twice daily on weekdays and once daily on weekends or public holidays for moribundity and mortality. All animals were observed daily for clinical signs, and a detailed clinical examination was performed weekly. During the acclimatization phase, all animals were weighed at least weekly before food distribution and food intake was measured for a minimum of 5 consecutive days before start of treatment. All animals were also subjected to an ophthalmological examination, hematology and clinical chemistry examinations, and urinalysis once during the acclimatization phase. Animals were selected and randomized for use in the study on the basis of acceptable findings from physical and ophthalmological examinations, body weight (within 20% of the mean weight for each sex), food consumption, clinical chemistry, hematology, and urinalysis. Animals were allocated to dosage groups using a dedicated computer system (Pristima System, version 6.3.2 Build 17, Xybion Corp.) in order to ensure a similar body weight distribution among groups of each sex, while ensuring full siblings were not placed in the same treatment group.

The dose levels used in this study were set on the basis of the results observed in the 28-day dog study (see data point 5.3.1/03), in which animals were administered BCS-CN88460 via the diet at concentrations of 300, 1000, and 3000 ppm. In this study, no treatment-related changes were observed at 300 ppm in either males or females. At 1000 ppm, alkaline phosphatase was increased in one female, while hepatocellular glycogen accumulation was decreased in one female and one male. At 3000 ppm, body weight loss was observed in both males and one of the two females, along with reduced food consumption. Alkaline

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phosphatase was increased in one male and in both females, while total cholesterol was decreased in one male and one female. Absolute and relative liver weights were increased in both males and females, and at histopathological examination the liver showed centrilobular to panlobular hypertrophy and decreased hepatocellular glycogen accumulation in both males and females, while brown pigment was observed in Kupffer cells in females.

Based on these observations, the dose levels chosen for the 90-day study in the male and female dog were 170, 500, and 1500 ppm.

3. Diet preparation and analysis

The test item was ground to a fine powder before the appropriate amount (weight:weight concentration) of test item was incorporated into the ground diet by dry mixing to provide the required dietary concentrations of 170, 500, or 1500 ppm. Two batches at each dose level were prepared to provide sufficient treated diet for the duration of the study. Each test diet mixture was stored at room temperature and issued to the animal unit in polyethylene bags placed in light-resistant containers.

The homogeneity of the test item in the diet was verified before the start of the study for the lowest and highest concentration, to demonstrate adequate formulation procedures. The mean values obtained from the homogeneity check were taken as measured concentrations. The dietary concentration of the test item in the diet was verified for all concentrations in all preparations from the first and second formulations.

The homogeneity analysis demonstrated that the concentration was at 88-96% of the nominal concentration, while concentration analysis yielded 89-101% of the nominal concentration. These results were considered to be acceptable for use on the current study.

4. Statistics

Statistical analyses were carried out using Pristima, version 6.3.2 build 17, Xybion Corp. Means and standard deviations were calculated for each group. All statistical analyses were carried out separately for males and females. Group means were compared at least at the 5% level of significance.

For body weight change parameters, terminal body weight, absolute and relative organ weight, specific hematology parameters, quantitative urinalysis parameters, and clinical chemistry parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight and food consumption parameters, and specific hematology parameters, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq$

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0.05), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.
If one or more group variances were equal to 0, means were compared using non-parametric procedures.

C. METHODS

1. Observations

Observed clinical signs were recorded at least once daily throughout the study, and any deviation from normal was recorded in respect to the nature and severity of the deviation. Daily examination of the kennels was also recorded for vomitus, diarrhea, or blood.

An additional detailed clinical examination was performed in an open area approximately weekly throughout the study. Recording of clinical signs included but were not limited to changes in general behavior and appearance, skin and fur, teeth and gums, eyes, ears, mucous membranes, gait, posture, and response to handling.

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

A detailed physical examination was performed on all dogs once during the acclimatization phase and monthly during the study. The physical examination included but was not limited to fur and skin, eyes, ears, teeth, gums, mucous membranes, rectal temperature, gait, stance, general behavior, chest including heart and respiratory rate, abdomen including palpation, external genitalia, and mammary glands. Also evaluated during the physical examination were:

- Mental state (level of consciousness, behavioral change)
- Posture
- Gait and motor function
- Muscle tone
- Postural reactions (tactile and visual placing, conscious proprioceptive positioning such as knuckling; hopping, wheelbarrowing)
- Spinal nerve reflexes (patellar; withdrawal (flexor), pelvic and thoracic limb; perianal; panniculus)
- Sensation (deep and superficial pain)
- Cranial nerve reflexes (general examination of the head; direct and indirect pupillary light; palpebral, i.e. blink and corneal; menace)

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2. **Body weight**
Body weights were measured prior to feeding at least weekly during the acclimatization phase and treatment period, and before final necropsy to determine terminal body weight.

3. **Food consumption and compound intake**
Food intake was measured for a minimum of five consecutive days immediately before start of treatment. The weight of food supplied to each animal and that remaining were recorded daily throughout the treatment period. From these records, the mean weekly consumption was calculated for each dog. Food spillage was also recorded.

The group mean achieved dosage for each sex, expressed as mg/kg bw/day, as calculated for each week and the overall mean was subsequently derived for weeks 1-13. This was calculated from the dietary test item concentration, food consumption, and body weight data.

4. **Ophthalmology**
During the acclimatization phase and again at the end of treatment, all animals were subjected to an ophthalmological examination after instillation of an atropinic agent. Each eye was examined by means of a slit lamp and an indirect ophthalmoscope. In the case of treatment-related effects being identified, photographic records were made and kept in the study file.

5. **Hematology and clinical chemistry, and bioanalytical examination**
Once before dosing, on study days 45 or 46, and on study days 81, 84, or 86, blood samples were taken from all animals in all groups by puncture of the jugular or other suitable vein. Blood was collected on EDTA for hematology, clot activator for serum for clinical chemistry, and sodium citrate for coagulation parameters. A blood smear was prepared for each animal and stained with May-Gruenwald-Giemsa, and was examined only if hematology results were abnormal. Standard hematology and clinical chemistry parameters were measured.

At the end of the study, a blood sample was collected from the jugular or other suitable vein of all surviving animals in each treated group before food distribution and approximately four hours (time of peak plasma concentration of BCS-CN88460, as determined in a previous study) and seven hours after food distribution. A blood sample was collected from two surviving animals per sex in the control group before food distribution only. Plasma was prepared by centrifugation from blood collected into heparinized vials and was stored as needed in the dark at approximately -20°C until shipment with dry ice for analysis.

6. **Urinalysis**
Once before dosing and on study days 43 or 45, and 85, overnight urine sample were collected from all animals in all groups. During urine collection dogs were left overnight without water. In the absence of voluntary voiding after a maximum of 2 attempts, urine was collected by transurethral catheterization. Urinary volume was not recorded in animals which were catheterized. Standard urinalysis parameters were measured.

7. **Sacrifice and pathology**
On study days 92 to 95, all animals from all groups were sacrificed. All animals were tranquilized by intramuscular injection of a combination of the tranquilizer zolazepam with the dissociative anesthetic tiletamine, followed by intravenous injection of pentobarbital. Animals were then exsanguinated and necropsied. All animals were diet-fasted prior to sacrifice. Necropsy included examination of the external surfaces, all orifices, and all major organs, tissues, and body cavities. Macroscopic abnormalities were recorded, sampled, and examined microscopically.

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Table 5.3.2/03-1: Organs and tissues which were sampled and / or weighed at necropsy

	Digestive system		**Cardiovasc. / Hemat.**		**Neurologic**
X	Tongue	X	Aorta*	XX	Brain with cerebellum*+
X	Submandibular (salivary) gland*	XX	Heart*+		
		X	Bone marrow, sternum*	X	Sciatic nerve*
X	Esophagus*	X	Lymph node, mesenteric*	X	Spinal cord (cervical, thoracic, lumbar)*
X	Stomach*				
X	Duodenum*	X	Lymph node, retropharyngeal*	X	Eyes*
X	Jejunum*			X	Optic nerves*
X	Ileum*	XX	Spleen*+	XX	Pituitary gland*
X	Cecum*	XX	Thymus		
X	Colon*				**Glandular**
X	Rectum*		**Urogenital**	XX	Adrenal gland*+
XX	Liver*+	XX	Kidney*+	X	Parathyroid gland*
X	Pancreas*	X	Urinary bladder*	XX	Thyroid gland* (weighed with parathyroid gland)+
X	Gallbladder	XX	Testis*+		
		XX	Epididymis*		
	Respiratory	XX	Prostate gland*		
X	Trachea*	X	Oviduct*		**Other**
X	Lung*	XX	Ovary*+	X	Bone (sternum)
X	Pharynx*	XX	Uterus (with cervix)*+	X	Skeletal muscle
X	Larynx*	X	Mammary gland*	X	Skin*
		X	Vagina	X	All gross lesions and masses
				X	Articular surface (femorotibial joint)

* recommended for 90-day oral non-rodent studies based on US EPA guideline 870.3150
\+ organ weights required for non-rodent studies based on US EPA guideline 870.3150
X tissues were collected for histological examinations
XX organs were weighed fresh at scheduled sacrifice only with paired organs weighed together, and collected for histological examination

For sacrificed animals, a bone marrow smear was prepared from one rib and stained with May-Gruenwald Giemsa, but not examined as no treatment-related changes were observed in bone marrow histology.

Samples were fixed by immersion in 10% neutral buffered formalin with the exception of the eye, optic nerve, epididymis, and testis, which were fixed in Davidson's fixative. Histological sections were prepared and stained with hematoxylin and eosin from all organs and tissue samples from all animals in all treatment groups.

II. RESULTS AND DISCUSSION

A. CLINICAL SIGNS AND MORTALITY

1. Clinical signs of toxicity and physical examination

The only treatment-related clinical sign was an increase in salivation at 1500 ppm in two of four females, at five occasions for one animal and seven occasions for the other animal.

There were no treatment-related changes noted at physical examination in either males or females, except for increased salivation noted for one high-dose female on day 91. Rectal temperature recordings in all animals revealed no treatment-related changes.

2. Mortality

There were no mortalities during the study.

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B. BODY WEIGHT AND BODY WEIGHT GAIN

At 170 and 500 ppm, mean body weight parameters were not affected by treatment in either males or females. At 1500 ppm, there was no effect of treatment on body weight or body weight gain during the first half of the study. However, during the end of the study mean body weight was decreased by 7% in males and 8% in females, and overall mean body weight gain was decreased by 39% relative to controls in both sexes.

Table 5.3.2/03-2: Mean body weight and cumulative body weight gain in male and female dogs administered BCS-CN88460 via the diet for 90 days

		BCS-CN88460, dietary concentration in ppm							
		Males				Females			
Parameter	Day	0	170	500	1500	0	170	500	1500
Body weight, kg	1	7.03	7.00	7.15	7.20	6.53	6.53	6.73	6.65
	8	7.35	7.25	7.53	7.45	6.80	6.88	6.85	6.80
	92	9.00	8.48	8.73	8.40	8.35	8.03	8.13	7.70
Body weight gain, kg	1-8	0.33	0.25	0.38	0.25	0.18	0.35	0.13	0.15
	1-92	1.98	1.48	1.58	1.20**	1.73	1.50	1.40	1.05

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

C. FOOD CONSUMPTION AND COMPOUND INTAKE

There was no effect of treatment on food consumption in either males or females in any group.

The mean achieved dose levels of BCS-CN88460 in this study are shown in Table 5.3.2/03-3.

Table 5.3.2/03-3: Mean achieved dose levels of BCS-CN88460, in mg/kg bw/day, in male and female rats administered the compound via the diet

	BCS-CN88460, dietary concentration in ppm					
	Males			Females		
	170	500	1500	170	500	1500
Weeks 1-13	5.5	15.9	50.4	5.5	16.2	54.0

D. BLOOD ANALYSES

1. Hematology

There were no treatment-related changes in either males or females in any dose group.

2. Clinical Chemistry

The only changes in clinical chemistry parameters which were considered to be related to treatment were an increase in alkaline phosphatase in both males and females at 1500 ppm and a decrease in bilirubin in both males and females (statistically significant only in males, at 500 and 1500 ppm). These findings are consistent with the liver being the primary target organ of BCS-CN88460.

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Table 5.3.2/03-4: Mean values for total bilirubin, total cholesterol, and albumin in male and female dogs administered BCS-CN88460 via the diet for 90 days

		BCS-CN88460, dietary concentration in ppm			
		Males			
	Week	0	170	500	1500
Alkaline phosphatase IU/L	PS	84.0	86.3	87.5	93.0
	7	104.5	136.5	139.8	217.5*
	12-13	89.3	135.5	142.3	213.5*
Total bilirubin µmol/L	PS	0.65	0.88	0.53	0.33
	7	0.78	0.90	0.40	0.45
	12-13	0.75	0.70	0.33	0.30
		Females			
Alkaline phosphatase IU/L	PS	70.8	76.0	118.0	123.5
	7	103.3	122.3	176.3	291.3*
	12-13	103.5	128.0	171.8	284.0*
Total bilirubin µmol/L	PS	0.68	0.93	0.73	1.03
	7	0.73	0.33	0.68	0.48
	12-13	0.73	0.33	0.40	0.33

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3. Urinalysis

There were no treatment-related changes in either males or females in any dose group.

4. Bioanalytical measurements

As measured in week 12, concentrations of BCS-CN88460 in the blood of treated animals increased in a dose-related manner. The greatest concentration in each treatment group was observed at four hours after food distribution, with plasma concentrations of BCS-CN88460 below or only slightly above the limit of quantification at the pre-feeding measurement.

Table 5.3.2/03-5: Concentrations of BCS-CN88460 in plasma in male and female dogs administered BCS-CN88460 via the diet for up to 90 days

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Parameter	0	170	500	1500	0	170	500	1500
Pre-feeding	< LOQ	< LOQ	< 0.012	0.027	< LOQ	< LOQ	< LOQ	0.040
4h after feeding	-	0.053	0.184	1.528	-	< 0.048	0.147	1.280
7h after feeding	-	0.036	0.124	1.030	-	< 0.044	0.119	0.825

E. SACRIFICE AND PATHOLOGY

1. Organ weight

At 1500 ppm, terminal body weight was decreased in both males and females, although this decrease was not statistically significant.

At 1500 ppm, absolute and relative liver weight were increased in males, although only the change in relative liver weight achieved statistical significance. In females, absolute liver weight and liver weight as a percentage of body weight were slightly increased, although this increase did not achieve statistical significance. Neither absolute nor relative liver weight were affected at either 500 or 170 ppm in either males or females.

There were no other treatment-related effects on organ weights in either sex.

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2. Gross and histopathology

There were no macroscopic findings which were considered to be related to treatment in either sex.

The only histopathological finding which was considered to be related to treatment was an increase in centrilobular hepatocellular hypertrophy in three of four males; in two animals, this was associated also with eosinophilic intracytoplasmic vacuoles. These changes were correlated with statistically significantly increased liver weights as well as the changes in alkaline phosphatase and total bilirubin shown in Table 5.3.2/03-4 above.

Table 5.3.2/03-6: Terminal body weight, mean absolute and relative liver weight, and treatment-related liver histopathological findings in male and female dogs administered BCS-CN88460 via the diet for 90 days

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	170	500	1500	0	170	500	1500
Terminal body wt, kg	8.93	8.50	8.68	8.38	8.38	7.95	8.08	7.65
Liver wt, g	252.2	269.4	284.0	338.5	279.4	278.3	291.1	308.1
Liver wt, % body wt	2.83	3.16	3.28	4.04**	3.34	3.48	3.60	4.03
Liver wt, % brain wt	340.4	344.1	383.3	460.7*	422.3	395.0	396.3	431.5
Hepatocellular hypertrophy, centrilobular								
Minimal	0	0	0	3	0	0	0	0
Slight	0	0	0	0	0	0	0	0
Total	0	0	0	3	0	0	0	0
Intracytoplasmic eosinophilic inclusion								
Total	0	0	0	2	0	0	0	0

Significant at * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.

F. DEFICIENCIES

There are no deficiencies noted in this study.

III. CONCLUSIONS

Based on the increase in absolute and relative liver weight in both males and females (as much as 43%), increased mean alkaline phosphatase, and microscopic changes in the liver of male dogs, the NOAEL in both males and females was established at 500 ppm, approximately 15.9 mg/kg bw/day in males and 16.2 mg/kg bw/day in females.

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Report:	KCA 5.3.2/04; [redacted]; 2017; M-601188-01-1
Title:	BCS-CN88460 - Chronic toxicity study in the dog by dietary administration
Report No.:	SA 14092
Document No.:	M-601188-01-1
Guideline(s):	OECD guideline 452 (September, 2009) EEC Directive 88/302/EEC, Method B.30 (1992) US EPA, OCSPP guideline number 870.4100 MAFF in Japan 12 Nousan N°8147 (November, 2000)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

BCS-CN88460 was administered to groups of four male and four female beagle dogs at dietary concentrations of 0, 150, 600, or 1800 ppm (equating to 0, 4.2, 18.8, and 60.2 mg/kg bw/day in males and 0, 4.2, 17.6, and 49.8 mg/kg bw/day in females) for at least 52 weeks. Each animal was checked for ill health, moribundity, and mortality twice daily or once daily on weekends and public holidays during the acclimatization phase and throughout the study. Observed clinical signs were recorded dailly. A detailed clinical examination was performed approximately weekly and a detailed physical examination was carried out on a monthly basis. Food consumption was recorded daily throughout the study and body weight was measured weekly and prior to necropsy. Ophthalmological examination was performed during the acclimatization phase and at the end of the study. Blood analysis (hematology and clinical chemistry) and urinalysis were performed once during the acclimatization phase, at months 4 and 6, and at the end of the study. Blood samples were also collected on all animals for bioanalytical examination at the end of the study. All animals were subjected to a detailed necropsy. Selected organs were weighed and a range of tissues were taken and processed for histopathological examination.

Up to the highest dietary concentration of 1800 ppm, BCS-CN88460 induced no treatment-related mortalities, and no treatment-related changes at physical and ophthalmological examination, hematology determination, or urinalysis.

One low dose group female was killed for human reasons on day 176 due to a congenital anomaly (persistent fourth right aortic arch) that had significantly affected the clinical condition of this dog.

At the end of the study (month 12), the bioanalytical examination showed a dose-related increase in mean plasma concentrations of BCS-CN88460 and metabolites BCS-CX99798 and BCS-CX99799, with essentially similar concentrations observed between the two sexes at the three time points investigated and peak concentrations noted 4 or 7 hours after food distribution.

<u>At 1800 ppm (60.2 and 49.8 mg/kg bw/day in males and females, respectively)</u>

The only treatment-related clinical signs consisted of increased salivation noted at the daily cage-side observation and / or weekly detailed clinical examination for one high dose male at 17 occasions throughout the study.

Mean body weight parameters were affected during the first week of the study, where a reduced mean body weight gain was noted in males (0.03 kg compared to a gain of 0.15 kg in controls), and a mean body weight loss of 0.15 kg was noted in females compared to a gain of 0.05 kg in controls. Thereafter, no statistically significant changes were noted in mean cumulative body weight gain was stastistically significantly reduced on a few occasions during the study compared to controls.

The mean weekly food consumption was reduced by 25% during the first week of the study in females compared to controls. This effect persisted during the study and led to an overall reduction by 10% in mean food consumption between days 1 and 364 in females, compared to controls. Mean food consumption was unaffected by treatment throughout the study in males.

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Clinical chemistry determination revealed incresaed mean alkaline phosphatase activities in both sexes on one or more occasions when compared to controls.

At necropsy, mean absolute and relative liver weights were statistically significantly higher in males when compared with controls and were unaffected in females. At macroscopic observation, enlarged liver was noted in 3/4 males and 1/4 females. At the microscopic examination, minimal to moderate centrilobular hepatocellular hypertrpohy was observed in the liver of all males and females, together with Kupffer cell pigmentation in two males and one female, single cell necrosis in one female, and cytoplasmic hepatocellular changes (eosinophilic intracytoplasmic inclusions) in one male. The liver changes were considered to be adverse, as they were correlated with statistically significantly higher liver weights in males and relevant changes at the clinical chemistry determination in both sexes.

At 600 ppm (18.8 and 17.6 mg/kg bw/day in males and females, respectively)
Clinical chemistry determination revealed increased mean alkaline phosphatase activities in both sexes on one or more occasions when compared to controls.

At necropsy, macroscopic observation showed enlarged liver in 1/4 females. At microscopic examination, minimal centrilobular hepatocellular hypertrophy was observed in 2/4 males and 3/4 females. The liver changes were considered to be adverse as they were correlated with relevant changes at clinical chemistry in both sexes.

At 150 ppm (4.2 mg/kg bw/day in both males and females)
There were no treatment-related changes in either sexes.

In conclusion, a dietary concentration of 150 ppm (4.2 mg/kg bw/day in both sexes) administered to beagle dogs for one year was considered to be a No Observed Effect Level (NOEL) in both sexes.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	beige solid
Lot / Batch #:	2013-006492
Purity:	94.2%
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	dog
Strain:	beagle
Age:	8-9 months old
Weight at dosing:	5.6-8.3 kg, males; 5.5-8.7 kg, females
Source:	[redacted]
Acclimation period:	25 days
Diet:	[redacted], France
Water:	Filtered and softened tap water from the municipal water supply, ad libitum

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Housing:	Individually in stainless steel kennels with a floor surface area of 2m2 during the day; where possible, they were pair-housed overnight by temporary opening of the partitions between 2 dogs from the same sex and dose group. Each week, supervised exercise was permitted in inside runs during cleaning of cages. During the exercise, dogs were separated by sex and dose group.
Environmental conditions:	
Temperature:	18-21°C
Humidity:	40-70%
Air changes:	Target of 15 per hour
Photoperiod:	12 hours dark, 12 hours light

B. STUDY DESIGN

1. **In life dates:** 23 October 2014-20 November 2015

2. **Animal assignment and treatment**
Shortly after arrival, all dogs were examined by a veterinarian for signs of ill health and were subjected to a detailed clinical examination to assess their physical and behavioral status. During the acclimatization phase, animals were checked twice daily on weekdays and once daily on weekends or public holidays for moribundity and mortality. All animals were observed daily for clinical signs, and a detailed clinical examination was performed weekly. During the acclimatization phase, all animals were weighed at least weekly before food distribution and food intake was measured for a minimum of 5 consecutive days before start of treatment. All animals were also subjected to an ophthalmological examination, hematology and clinical chemistry examinations, and urinalysis once during the acclimatization phase. Animals were selected and randomized for use in the study on the basis of acceptable findings from physical and ophthalmological examinations, body weight, food consumption, clinical chemistry, hematology, and urinalysis. Animals were allocated to dosage groups using a dedicated computer system (Pristima System, version 6.3.2 Build 17, Xybion Corp.) in order to ensure a similar body weight distribution among groups of each sex, while ensuring full siblings were not placed in the same treatment group.

The dose levels used in this study were set on the basis of the results observed in the 90-day dietary study (see data point 5.3.2/03), in which animals were administered BCS-CN88460 via the diet at concentrations of 0, 170, 500, and 1500 ppm. In that study, there were no adverse findings at either 170 or 500 ppm. At 1500 ppm, there was a biologically significant reduction in overall mean body weight gain in both males and females. Alkaline phosphatase was increase at weeks 7 and 13, with a greater effect in females than in males. Liver weight was increased in males, accompanied by an increase in the incidence of centrilobular hepatocellular hypertrophy.

Based on these observations, the dose levels chosen for this one-year dog study were 0, 150, 600, and 1800 ppm.

3. **Diet preparation and analysis**
The test item was ground to a fine powder before the appropriate amount (weight/weight concentration) of test item was incorporated into the ground diet by dry mixing to provide the required dietary concentrations of 150, 600, and 1800 ppm. Nine batches at each dose level were prepared to provide sufficient treated diet for the duration of the study. Each test diet mixture was stored at room temperature and issued to the animal unit in polyethylene bags placed in light-resistant containers.

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The stability of the test item in diet at 100 and 5000 ppm was determined in a previous study under storage and use conditions similar to those of the current study, where it was demonstrated that BCS-CN88460 was stable for up to 76 days at room temperature after mixing into dry diet. In addition, the stability of BCS-CN88460 at concentrations of 100 and 5000 ppm in the moistened diet as distributed to the animals was demonstrated for a period of 4 hours, covering the time of food preparation and distribution.

The homogeneity of the test item in the diet was verified for all concentrations on the first preparation from the first formulation, and for the lowest and highest concentrations on the first preparation from the fifth formulation, to demonstrate adequate formulation procedures. The concentration of the test item in the diet was verified for all concentrations on all preparations from the first, third, fifth, seventh, and ninth formulations. The mean values obtained from the homogeneity check on the first and fifth formulations were taken as measured concentration.

The homogeneity analysis showed that the concentration was at 88-101% of nominal concentration, while the concentration analysis yielded 93-99% of nominal concentrations. As these were within the in-house target range of 85-115% of nominal concentrations, the formulations were considered to be acceptable for use on the current study.

4. Statistics

Statistical analyses were carried out using Pristima, version 6.3.2 build 17, Xybion Corp. Means and standard deviations were calculated for each group. All statistical analyses were carried out separately for males and females. Group means were compared at least at the 5% level of significance.

For body weight change parameters, terminal body weight, absolute and relative organ weight, specific hematology parameters, quantitative urinalysis parameters, and clinical chemistry parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight and food consumption parameters, and specific hematology parameters, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-

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sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.
If one or more group variances were equal to 0, means were compared using non-parametric procedures.

C. METHODS

1. Observations

Observed clinical signs were recorded at least once daily throughout the study, and any deviation from normal was recorded in respect to the nature and severity of the deviation. Daily examination of the kennels was also recorded for vomitus, diarrhea, or blood.

An additional detailed clinical examination was performed in an open area approximately weekly throughout the study. Recording of clinical signs included but were not limited to changes in general behavior and appearance, skin and fur, teeth and gums, eyes, ears, mucous membranes, gait, posture, and response to handling.

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

A detailed physical examination was performed on all dogs once during the acclimatization phase and monthly during the study. The physical examination included but was not limited to fur and skin, eyes, ears, teeth, gums, mucous membranes, rectal temperature, gait, stance, general behavior, chest including heart and respiratory rate, abdomen including palpation, external genitalia, and mammary glands. Also evaluated during the physical examination were:

- Mental state (level of consciousness, behavioral change)
- Posture
- Gait and motor function
- Muscle tone
- Postural reactions (tactile and visual placing, conscious proprioceptive positioning such as knuckling, hopping, wheelbarrowing)
- Spinal nerve reflexes (patellar, withdrawal (flexor), pelvic and thoracic limb; perianal, panniculus)
- Sensation (deep and superficial pain)
- Cranial nerve reflexes (general examination of the head; direct and indirect pupillary light; palpebral, i.e. blink and corneal; menace)

2. Body weight

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

3. Food consumption and compound intake

Food intake was measured for a minimum of five consecutive days immediately before start of treatment. The weight of food supplied to each animal and that remaining were recorded

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daily throughout the treatment period. From these records, the mean weekly consumption was calculated for each dog. Food spillage was also recorded.

The group mean achieved dosage for each sex, expressed as mg/kg bw/day, as calculated for each week and the overall mean was subsequently derived for weeks 1-52. This was calculated from the dietary test item concentration, food consumption, and body weight data.

4. **Ophthalmology**
During the acclimatization phase and again at the end of treatment, all animals were subjected to an ophthalmological examination after instillation of an atropinic agent. Each eye was examined by means of a slit lamp and an indirect ophthalmoscope. In the case of treatment-related effects being identified, photographic records were made and kept in the study file.

5. **Hematology and clinical chemistry, and bioanalytical examination**
Once before dosing, on study days 94 or 95, 162 or 163, and 358 or 359, blood samples were taken from all surviving animals in all groups by puncture of the jugular vein. Blood was collected on EDTA for hematology, clot activator for serum for clinical chemistry, and sodium citrate for coagulation parameters. A blood smear was prepared for each animal and stained with May-Gruenwald-Giemsa, and was examined only if hematology results were abnormal. Standard hematology and clinical chemistry parameters were measured.

At the end of the study, a blood sample was collected from the jugular or other suitable vein of all surviving animals in each treated group before food distribution and approximately four hours (time of peak plasma concentration of BCS-CN88460, as determined in a previous study) and seven hours after food distribution. A blood sample was collected from two surviving animals per sex in the control group before food distribution only. Plasma was prepared by centrifugation from blood collected into heparinized vials and was stored as needed in the dark at approximately -20°C until shipment with dry ice for analysis.

6. **Urinalysis**
Once before dosing and on study days 100 or 101, 158 or 159, and 362 or 365, overnight urine samples were collected from all animals in all groups. During urine collection dogs were left overnight without water. In the absence of voluntary voiding after a maximum of 2 attempts, urine was collected by transurethral catheterization. Urinary volume was not recorded in animals which were catheterized. Standard urinalysis parameters were measured.

7. **Sacrifice and pathology**
On study days 366 to 369, all animals from all groups were sacrificed. All animals were tranquilized by intramuscular injection of a combination of the tranquilizer zolazepam with the dissociative anesthetic tiletamine, followed by intravenous injection of pentobarbital. Animals were then exsanguinated and necropsied. All animals were diet-fasted prior to sacrifice. Necropsy included examination of the external surfaces, all orifices, and all major organs, tissues, and body cavities. Macroscopic abnormalities were recorded, sampled, and examined microscopically.

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Table 5.3.2/04-1: Organs and tissues which were sampled and / or weighed at necropsy

	Digestive system		**Cardiovasc. / Hemat.**		**Neurologic**
X	Tongue	X	Aorta*	XX	Brain with cerebellum*+
X	Submandibular (salivary) gland*	XX	Heart*+		
		X	Bone marrow, sternum*	X	Sciatic nerve*
X	Esophagus*	X	Lymph node, mesenteric*	X	Spinal cord (cervical, thoracic, lumbar)*
X	Stomach*				
X	Duodenum*	X	Lymph node, retropharyngeal*	X	Eyes*
X	Jejunum*			X	Optic nerves*
X	Ileum*	XX	Spleen*+	XX	Pituitary gland*
X	Cecum*	XX	Thymus		
X	Colon*				**Glandular**
X	Rectum*		**Urogenital**	XX	Adrenal gland*+
XX	Liver*+	XX	Kidney*+	X	Parathyroid gland*
X	Pancreas*	X	Urinary bladder*	XX	Thyroid gland* (weighed with parathyroid gland)+
X	Gallbladder	XX	Testis*+		
		XX	Epididymis*+		
	Respiratory	XX	Prostate gland*		
X	Trachea*	X	Oviduct*		**Other**
X	Lung*	XX	Ovary*+	X	Bone (sternum)
X	Pharynx*	XX	Uterus (with cervix)*+	X	Skeletal muscle
X	Larynx*	X	Mammary gland*	X	Skin*
		X	Vagina	X	All gross lesions and masses
				X	Articular surface (femorotibial joint)

* required for chronic studies based on US EPA guideline 870.4100
\+ organ weights required for non-rodent studies based on US EPA guideline 870.4100
X tissues were collected for histological examinations
XX organs were weighed fresh at scheduled sacrifice only, with paired organs weighed together, and collected for histological examination

For sacrificed animals, a bone marrow smear was prepared from one rib and stained with May-Gruenwald Giemsa, but not examined as no treatment-related changes were observed in bone marrow histology.

Samples were fixed by immersion in 10% neutral buffered formalin with the exception of the eye, optic nerve, epididymis and testis, which were fixed in Davidson's fixative. Histological sections were prepared and stained with hematoxylin and eosin from all organs and tissue samples from all animals in all treatment groups.

II. RESULTS AND DISCUSSION

A. CLINICAL SIGNS AND MORTALITY

1. Clinical signs of toxicity and physical examination

The only treatment-related clinical sign was an increase in salivation at 1800 ppm in one male on 17 occasions throughout the study.

There were no treatment-related changes noted at physical examination in either males or females.

2. Mortality

There were no treatment-related mortalities during the study. One female at 150 ppm was killed for humane reasons on study day 176. This animal was diagnosed, after body weight loss and progressive reduction in food consumption, with a congenital anomaly responsible

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for the clinical condition of the animal. Necropsy revealed a persistent fourth right aortic arch, leading to dilated esophagus with dilated stomach. Other observations included enlarged mesenteric lymph nodes and a cyst on the uterus. The in-life parameters for this animal were excluded from all analyses.

B. BODY WEIGHT AND BODY WEIGHT GAIN

At 150 and 600 ppm, mean body weight parameters were not affected by treatment in either males or females. At 1800 ppm, during the first week of the study, reduced mean body weight gain was observed in males, and mean body weight loss was observed in females. In females in this group, mean cumulative body weight gain was subsequently reduced on a few occasions during the study.

Table 5.3.2/04-2: Mean body weight and cumulative body weight gain in male and female dogs administered BCS-CN88460 via the diet for one year

		BCS-CN88460, dietary concentration in ppm							
		Males				Females			
Parameter	Day	0	150	600	1800	0	150	600	1800
Body weight, kg	1	7.08	7.05	6.93	6.98	7.40	8.10	7.10	7.13
	9	7.23	7.25	7.05	7.00	7.45	8.17	7.23	7.00
	93	8.60	8.55	8.05	8.23	8.75	9.73	8.13	7.90
	184	9.15	8.90	8.45	8.60	9.30	10.30	8.73	8.40
	361	9.65	9.10	8.60	8.83	9.95	11.10	9.08	8.85
Body weight gain, kg	1-9	0.019	0.025	0.016	0.003	0.05	0.07	0.13	-0.013
	1-93	1.53	1.50	1.13	1.25	1.33	1.63	1.03	0.78
	1-184	2.08	1.85	1.53	1.63	1.90	2.20	1.63	1.28
	3-361	2.58	2.05	1.68	1.85	2.55	3.00	1.98	1.73

C. FOOD CONSUMPTION AND COMPOUND INTAKE

There was no effect of treatment on food consumption in either males or females at 150 or 600 ppm, nor was food consumption at 1800 ppm in males affected by dietary administration of BCS-CN88460. In females at 1800 ppm food consumption was decreased by 25% during the first week of the study, and overall food consumption between days 1 and 364 was reduced by 10% compared to controls.

The mean achieved dose levels of BCS-CN88460 in this study are shown in Table 5.3.2/04-3.

Table 5.3.2/04-3: Mean achieved dose levels of BCS-CN88460, in mg/kg bw/day, in male and female dogs administered the compound via the diet for one year

	BCS-CN88460, dietary concentration in ppm					
	Males			Females		
	150	600	1800	150	600	1800
Weeks 1-13	5.0	21.7	65.0	5.1	20.7	55.5
Weeks 1-52	4.2	18.8	60.2	4.2	17.6	49.8

D. BLOOD ANALYSES

1. Hematology

There were no treatment-related changes in either males or females in any dose group.

2. Clinical Chemistry

The only changes in clinical chemistry parameters which were considered to be related to treatment were an increase in alkaline phosphatase in both males and females at 1800 ppm and a decrease in bilirubin in both males and females. These findings are consistent with the liver being the primary target organ of BCS-CN88460.

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Table 5.3.2/04-4: Mean values for total bilirubin, total cholesterol, and albumin in male and female dogs administered BCS-CN88460 via the diet for one year

		BCS-CN88460, dietary concentration in ppm			
		Males			
	Month	**0**	**150**	**600**	**1800**
Alkaline phosphatase IU/L	Pre-study	89.8	98.0	132.3	137.3
	4	69.8	87.3	156.5*	266.0**
	6	63.0	85.0	127.3	243.3**
	12	57.3	82.5	152.5	268.8**
Total bilirubin µmol/L	Pre-study	0.35	0.23	0.08	0.30
	4	0.98	0.55	0.45	0.25
	6	0.80	0.33	0.35	0.10
	12	0.78	1.13	0.80	0.30
		Females			
Alkaline phosphatase IU/L	Pre-study	116.0	114.0	96.3	82.0
	4	110.3	102.5	132.5	185.5
	6	105.0	83.0	144.5	176.5
	12	108.0	106.0	180.0	250.8*
Total bilirubin µmol/L	Pre-study	0.23	0.30	0.18	0.60
	4	1.10	0.63	0.65	0.40
	6	1.48	0.55*	0.45**	0.28**
	12	1.20	0.53	0.55	0.53

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3. Urinalysis

There were no treatment-related changes in either males or females in any dose group.

4. Bioanalytical measurements

As measured in month 12, concentrations of BCS-CN88460 and the metabolites BCS-CX99798 and BCS-CX99798 in the blood of treated animals increased in a dose-related manner. The greatest concentration in each treatment group was observed at four hours after food distribution, with plasma concentrations of BCS-CN88460 below or only slightly above the limit of quantification at the pre-feeding measurement.

Table 5.3.2/04-5: Concentrations of BCS-CN88460 and metabolites in plasma in male and female dogs administered BCS-CN88460 via the diet for one year

		BCS-CN88460, dieteary concentration in ppm					
		Males			Females		
		150	**600**	**1800**	**150**	**600**	**1800**
BCS-CN88460	Pre-feeding	< LOQ	0.013	< 0.048	< LOQ	< 0.016	0.145
	4h after feeding	< LOQ	0.108	2.540	< 0.02	0.225	1.365
	7h after feeding	< 0.011	0.115	1.795	< LOQ	< 0.231	0.603
BCS-CX99798	Pre-feeding	< LOQ	0.031	< 0.093	< LOQ	0.037	0.087
	4h after feeding	< LOQ	0.052	0.193	< 0.01	0.077	0.140
	7h after feeding	< 0.012	0.074	0.225	< 0.010	0.088	0.176
BCS-CX99799	Pre-feeding	< LOQ	0.129	0.114	< 0.01	0.101	0.183
	4h after feeding	< 0.011	0.225	0.553	< 0.020	0.305	0.385
	7h after feeding	< 0.018	0.225	0.433	< 0.012	0.254	0.380

For series with some of the individual values below the limit of quantification (LOQ) of 0.01 mg/L, the value of the LOQ was used for calculation of the group mean; the result was then expressed as "< mean value".

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E. SACRIFICE AND PATHOLOGY

1. Organ weight

Mean terminal body weight was decreased in all male treatment groups and in females at 600 and 1800 ppm. As there was no dose relationship and no statistical significance, this was considered not to be a treatment-related effect.

At 1800 ppm in males, absolute and relative liver weights were statistically significantly increased relative to controls. There was no effect on either absolute or relative liver weight in females at any dose level.
There were no other treatment-related effects on organ weights in either sex.

2. Gross and histopathology

Enlarged liver was noted in three of the four males and one of the four females at 1800 ppm, and in one of four females at 600 ppm. There were no other macroscopic findings which were considered to be related to treatment in either sex.

The only histopathological finding which was considered to be related to treatment was an increase in centrilobular hepatocellular hypertrophy in all animals at 1800 ppm as well as in two males and three females at 600 ppm. At 1800 ppm, Kupffer cell pigmentation was noted in two males and one female, along with single cell necrosis in one female and eosinophilic intracytoplasmic inclusion in one male. These changes were correlated with statistically significantly increased liver weights as well as the changes in alkaline phosphatase and total bilirubin shown in Table 5.3.2/04-4 above.

Table 5.3.2/04-6: Terminal body weight, mean absolute and relative liver weight, and treatment-related liver histopathological findings in male and female dogs administered BCS-CN88460 via the diet for one year

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	150	600	1800	0	150	600	1800
Terminal body wt, kg	9.68	9.10	8.56	8.58	10.03	11.10	9.03	8.88
Liver wt, g	241.8	215.5	266.2	346.2**	255.6	292.7	271.6	279.0
Liver wt, % body wt	2.52	2.38	3.12	3.96**	2.56	2.69	3.01	3.21
Liver wt, % brain wt	313.2	296.8	380.4	437.0**	345.7	367.4	366.5	393.0
Macro- and microscopic findings								
N examined	4	4	4	4	4	3	4	4
Enlarged liver	0	0	0	3	0	0	1	1
Hepatocellular hypertrophy, centrilobular								
Total	0	0	3	4	0	0	3	4
Intracytoplasmic eosinophilic inclusion								
Total	0	0	0	1	0	0	0	0
Kupffer cell pigmentation, focal								
Total	0	0	0	2	0	0	0	1
Single cell necrosis: focal								
Total	0	0	0	0	0	0	0	1

Significant at * $p \leq 0.05$ ** $p \leq 0.01$; *** $p \leq 0.001$.

F. DEFICIENCIES

There are no deficiencies noted in this study.

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III. CONCLUSIONS

Based on the increase in liver weight observed in males and in the incidence of enlarged liver in both sexes, on increases in alkaline phosphatase in both males and females, and on histopathological findings including centrilobular hepatocellular hypertrophy observed in both males and females, the NOEL for this one-year study in the dog with BCS-CN88460 is established at 150 ppm (4.2 mg/kg bw/day in both sexes).

CA 5.3.3 Other routes

Toxicological studies were not conducted by other means of administration than via the diet.

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CA 5.4 Genotoxicity testing

Table 5.4-1: Summary of Isoflucypram genotoxicity studies

Study	Concentrations of [Substance] tested	Result	Reference
***In vitro* assays**			
Ames microsomally-mediated reverse mutagenesis assay	0, 3, 10, 33, 100, 333, 1000, 2500, 5000 µg/plate	negative	[redacted] 2014; M-490251-01-1
Chromosomal aberrations study in human lymphocytes *in vitro*	Concentrations evaluated: Without S9, exp.1: 0, 7.8, 13., 23.9 µg/mL exp.2: 0, 2.0, 3.5, 6.1 µg/mL With S9, exp.1: 0, 13.6, 23.9, 41.8 µg/mL; exp.2: 0, 10.0, 20.0, 30.0, 40.0 µg/mL	clastogenic	[redacted] 2014; M-495536-01-1
V79 / HPRT mammalian mutagenicity study	Without S9, exp.1 (4 hours): 0, 4.0, 8.0, 16.0, 24.0, 32.0 µg/mL; exp. 2 (24 hours): 0, 8.0, 16.0, 32.0, 48.0, 64.0 µg/mL. With S9, exp.1 (4 hours): 0, 8.0, 16.0, 32.0, 48.0, 64.0 µg/mL; exp. 2 (4 hours): 0, 8.0, 16.0, 32.0, 48.0, 64.0, and 128.0 µg/mL	Negative	[redacted] 2014; M-488526-01-1
***In vivo* assay**			
Mouse micronucleus assay *in vivo*	0, 500, 1000, and 2000 mg/kg bw	Negative	[redacted]; 2014; M-485866-01-1

BCS-CN88460 was tested in the Ames microsomally-mediated S. typhimurium reverse mutagenesis assay, the *in vitro* chromosome aberration study using human lymphocytes, the *in vitro* V79 / HPRT gene mutation assay in Chinese hamster V79 cells, and an *in vivo* mouse micronucleus study. The Ames bacterial mutagenicity and V79 / HPRT mammalian mutagenicity studies were negative both with and without S9 enzymatic activating system. In the *in vitro* chromosome aberration study, there was a statistically significant increase in the incidence of chromosomal aberrations (both including and excluding gaps) in both the presence and the absence of S9 enzymatic activating system. There was no increase in the incidence of polyploid metaphases in any experimental conditions in that study.

In the *in vivo* mouse micronucleus, clinical signs of toxicity were observed after oral gavage administration (two administrations of 2000 mg/kg bw/day separated by 24 hours), but there was no effect of treatment on the ratio of polychromatic to normochromatic erythrocytes, nor was there any increase in the incidence of micronucleated polychromatic erythrocytes. Although plasma concentrations of BCS-CN88460 were not measured, results from the rat ADME study (see data point 5.1.1/03) indicate that the compound does reach rodent bone marrow after oral gavage administration. Furthermore, measurement of the compound and its metabolites in the mouse chronic study (see data point 5.5/02) showed that significant amounts of the metabolites BCS-CX99798 and BCS-CX99799 were detected after dietary administration. Thus, it can be considered that the test item was absorbed

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after oral gavage administration to male mice in the *in vivo* mouse micronucleus study and that it or its metabolites reached and thus exposed the bone marrow.

The weight of evidence approach shows that BCS-CN88460 is not genotoxic in *in vitro* or *in vivo* systems. This is supported by the absence of tumors in either the rat or mouse chronic studies.

CA 5.4.1 *In vitro* studies

Report:	KCA 5.4.1/01; [redacted]; 2014, M-490251-01-1
Title:	BCS-CN88460; technical: Salmonella typhimurium reverse mutation assay
Report No.:	1614801
Document No.:	M-490251-01-1
Guideline(s):	OECD 471 (1997) EC 440/2008 B13/14 (2008) EPA OPPTS 870.5100 (1998)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

This study was perfomed to investigate the potential of BCS-CN88460 to induce gene mutations according to the plate incorporation test (Experiment 1) and the pre-incubation test (Experiment 2) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102. The assay was performed in two independent experiments, both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at the concentrations of 0, 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate.

The test item precipitated in the overaly agar in the test tubes from 1900 to 5000 µg/plate. Precipitation of the test item in the overlay agar on the incubated agar plates was observed from 333 to 5000 µg/plate. The undissolved particles had no influence on the data recording.

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation with the exception of strain TA 1537, where minor toxic effects were observed at 1000 and 5000 µg/plate without metabolic activation in Experiment 1 and at 2500 and 5000 µg/plate with metabolic activation in Experiment 2.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-CN88460 at any dose level, neither in the presence nor in the absence of metabolic activation. There was also no tendency towards increased mutation rates with increased concentrations in the range below the generally acknowledged limit of biological relevance. Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, BCS-CN88460 is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	BCS-CN88460
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% (w/w)
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

2. Control materials:	
Negative:	None
Solvent / final concentration:	DMSO
Positive:	Without metabolic activation: Sodium azide (NaN_2, TA 1535, TA 100); 4-nitro-o-phenylene-diamine (4-NOPD, TA 1537, TA 98); methyl methane sulfonate (MMS, TA 102) With metabolic activation: 2-aminoanthracene (2-AA) for all strains

3. Activation:	
Source	Rat liver S9, from rats induced with phenobarbital and β-naphthoflavone
S9 mix composition:	10% S9 supernatant 8 mM $MgCl_2$ 33 mM KCl 5 mM glucose-6-phosphate 4 mM NADP In 100 mM sodium-ortho-phosphate buffer at pH 7.4

4. Test organisms: Salmonella typhimurium strains TA 98, TA 100, TA 102, TA 1535, TA 1537

5. Test concentrations:	
Preliminary cytotoxicity assay:	3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate
Mutation assays:	3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate

B. TEST PERFORMANCE

1. In life dates: 1-16 April 2014

2. Preliminary cytotoxicity assay

For each strain and dose level, including the controls, three plates were used. A volume of 100 µl of the test solution, solvent, or reference mutagen solution (positive control), 500 µl of the S9 mix (for tests with metabolic activation) or the S9 mix substitution buffer (for tests without metabolic activation), 100 µl of the bacterial suspension, and 2000 µl of overlay agar were mixed in a test tube and poured onto selective agar plates.

After solidification, the plates were incubated upside down for at least 48 hours at 37°C in the dark.

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Colonies were counted using the Petri Viewer Mk2 ([redacted]) with the software program Ames Study Manager (v. 1.21). The counter was connected to a PC with printer to print out the individual values and mean values of the plates for each concentration, together with standard deviations and enhancement factors as compared to the spontaneous reversion rates. Due to precipitation of the test item, the colonies were partly counted manually.

3. **Plate incorporation mutation assay**
For each strain and dose level, including the controls, three plates were used. A volume of 100 µl of the test solution, solvent, or reference mutagen solution (positive control), 500 µl of the S9 mix (for tests with metabolic activation) or the S9 mix substitution buffer (for tests without metabolic activation), 100 µl of the bacterial suspension, and 2000 µl of overlay agar were mixed in a test tube and poured onto selective agar plates.

After solidification, the plates were incubated upside down for at least 48 hours at 37°C in the dark.

Colonies were counted using the Petri Viewer Mk2 ([redacted]) with the software program Ames Study Manager (v. 1.21). The counter was connected to a PC with printer to print out the individual values and mean values of the plates for each concentration, together with standard deviations and enhancement factors as compared to the spontaneous reversion rates. Due to precipitation of the test item, the colonies were partly counted manually.

4. **Pre-incubation assay**
For each strain and dose level, including the controls, three plates were used. A volume of 100 µl of the test solution, solvent, or reference mutagen solution (positive control), 500 µl of the S9 mix (for tests with metabolic activation) or the S9 mix substitution buffer (for tests without metabolic activation), and 100 µl of the bacterial suspension were mixed in a test tube and incubated at 37°C for 60 minutes. After pre-incubation, 2.0 ml overlay agar at 45°C was added to each tube, and the mixture was poured on minimal agar plates.

After solidification, the plates were incubated upside down for at least 48 hours at 37°C in the dark.

Colonies were counted using the Petri Viewer Mk2 ([redacted]) with the software program Ames Study Manager (v. 1.21). The counter was connected to a PC with printer to print out the individual values and mean values of the plates for each concentration, together with standard deviations and enhancement factors as compared to the spontaneous reversion rates. Due to precipitation of the test item, the colonies were partly counted manually.

5. **Statistics**
According to the OECD guideline 471, statistical analysis of the data is not mandatory.

6. **Evaluation criteria**
The reverse mutation assay is considered acceptable if regular background growth is noted in the negative and solvent controls; if spontaneous reversion rates in the negative and solvent controls are within the range of the conducting laboratory's historical control data; if the positive control substances produce a significant increase in mutant colony frequencies; and if at least five analyzable dose levels are present with at least three dose levels showing no signs of bacteriotoxic effects (evident as a reduction in the number of revertants below the indication factor of 0.5).

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A test item is considered a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains (TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed. A dose-dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration, while an increase exceeding this threshold at only one concentration is considered to be biologically relevant if it is reproduced in a second experiment.

A dose-dependent increase in the number of revertant colonies below the threshold is considered an indication of mutagenic potential if it is reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls, such an increase is not considered biologically relevant.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations of concentration, homogeneity, or stability of the test item in the vehicle were not conducted.

B. PRELIMINARY CYTOTOXICITY ASSAY

The preliminary cytotoxicity assay did not show any toxicity of BCS-CN88460 to the bacterial strains used, and thus the results of this assay are presented as the plate incorporation assay.

C. MUTATION ASSAYS

In both the plate incorporation and the pre-incubation assays, BCS-CN88460 was tested at 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate. The test substance precipitated in the overlay agar in the test tubes from 1000 to 5000 µg/plate. Precipitation of the test item in the overlay agar on the incubated agar plates was observed from 333 to 5000 µg/plate. The undissolved particles had no influence on the data recording.

The plates incubated with BCS-CN88460 showed normal background growth up to 5000 µg/plate, both with and without S9 mix in all strains used. No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups either with or without metabolic activation, with the exception of strain TA 1537, where minor toxic effects were observed at 1000 and 5000 µg/plate without metabolic activation in the plate incorporation assay and at 2500 and 5000 µg/plate with metabolic activation in the pre-incubation assay.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-CN88460 at any concentration level in either the presence or the absence of metabolic activation.

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Table 5.4.1/01-1: Results from the plate incorporation assay in Salmonella typhimurium strains with BCS-CN88460

			Revertant colony counts (mean ± SD)				
S9	**Test item**	**Dose / plate**	**TA 1535**	**TA 1537**	**TA 98**	**TA 100**	**TA 102**
-	DMSO	-	13 ± 2	9 ± 2	20 ± 7	151 ± 20	445 ± 40
	Untreated	-	13 ± 2	9 ± 3	25 ± 5	170 ± 8	480 ± 14
	BCS-CN88460	3 µg	15 ± 2	7 ± 1	24 ± 8	149 ± 17	424 ± 17
		10 µg	15 ± 3	9 ± 1	23 ± 6	142 ± 13	475 ± 51
		33 µg	17 ± 3	9 ± 5	22 ± 5	157 ± 29	466 ± 25
		100 µg	17 ± 2	7 ± 2	24 ± 4	148 ± 18	456 ± 18
		333 µg	12 ± 4[PM]	8 ± 1[PM]	22 ± 5[PM]	163 ± 11[PM]	456 ± 19[PM]
		1000 µg	15 ± 1[PM]	2 ± 1[PM]	20 ± 3[PM]	152 ± 17[PM]	422 ± 12[PM]
		2500 µg	15 ± 1[PM]	5 ± 3[PM]	21 ± 2[PM]	180 ± 11[PM]	460 ± 23[PM]
		5000 µg	14 ± 2[PM]	4 ± 2[PM]	19 ± 2[PM]	175 ± 28[PM]	471 ± 7[PM]
	NaN3	10 µg	2970 ± 236			1729 ± 110	
	4-NOPD	10 µg			272 ± 19		
		50 µg		65 ± 15			
	MMS	2.0 µL					5413 ± 86
+	DMSO	-	13 ± 2	15 ± 2	34 ± 7	167 ± 32	552 ± 74
	Untreated	-	13 ± 2	19 ± 6	48 ± 1	178 ± 14	623 ± 4
	BCS-CN88460	3 µg	14 ± 1	19 ± 3	47 ± 4	161 ± 10	662 ± 8
		10 µg	13 ± 1	14 ± 2	35 ± 6	149 ± 22	656 ± 58
		33 µg	13 ± 2	18 ± 9	36 ± 6	150 ± 10	656 ± 87
		100 µg	10 ± 3	25 ± 2	41 ± 4	166 ± 10	621 ± 51
		333 µg	11 ± 2[PM]	13 ± 2[PM]	36 ± 2[PM]	158 ± 4[PM]	511 ± 24[PM]
		1000 µg	10 ± 2[PM]	10 ± 3[PM]	26 ± 3[PM]	156 ± 13[PM]	553 ± 8[PM]
		2500 µg	16 ± 1[PM]	18 ± 3[PM]	38 ± 10[PM]	156 ± 3[PM]	533 ± 13[PM]
		5000 µg	22 ± 1[PM]	14 ± 1[PM]	35 ± 3[PM]	162 ± 6[PM]	504 ± 12[PM]
	2-AA	2.5 µg	549 ± 15	307 ± 27	3304 ± 172	3722 ± 153	
		10.0 µg					3834 ± 153

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Table 5.4.1/01-2: Results from the pre-incubation assay in Salmonella typhimurium strains with BCS-CN88460

			Revertant colony counts (mean ± SD)				
S9	**Test item**	**Dose / plate**	**TA 1535**	**TA 1537**	**TA 98**	**TA 100**	**TA 102**
-	DMSO	-	17 ± 2	9 ± 3	23 ± 4	131 ± 10	388 ± 22
	Untreated	-	16 ± 3	9 ± 3	26 ± 3	195 ± 4	466 ± 11
	BCS-CN88460	3 µg	10 ± 2	10 ± 3	24 ± 4	122 ± 14	447 ± 28
		10 µg	12 ± 4	10 ± 2	24 ± 1	130 ± 3	409 ± 18
		33 µg	19 ± 4	8 ± 4	22 ± 6	134 ± 12	453 ± 22
		100 µg	14 ± 4	11 ± 5	26 ± 5	139 ± 11	427 ± 21
		333 µg	16 ± 4[PM]	15 ± 6[PM]	39 ± 4[PM]	144 ± 8[PM]	415 ± 13[PM]
		1000 µg	14 ± 3[PM]	9 ± 2[PM]	31 ± 2[PM]	144 ± 11[PM]	413 ± 10[PM]
		2500 µg	18 ± 4[PM]	7 ± 3[PM]	28 ± 3[PM]	128 ± 14[PM]	422 ± 14[PM]
		5000 µg	14 ± 3[PM]	9 ± 2[PM]	30 ± 7[PM]	116 ± 15[PM]	384 ± 30[PM]
	NaN3	10 µg	2796 ± 210			1720 ± 208	
	4-NOPD	10 µg			345 ± 38		
		50 µg		67 ± 16			
	MMS	2.0 µL					4324 ± 96
+	DMSO	-	11 ± 3	20 ± 2	38 ± 2	126 ± 7	589 ± 85
	Untreated	-	15 ± 5	20 ± 7	41 ± 13	169 ± 10	503 ± 54
	BCS-CN88460	3 µg	14 ± 6	23 ± 3	47 ± 3	122 ± 8	537 ± 59
		10 µg	15 ± 1	20 ± 3	39 ± 8	126 ± 14	492 ± 18
		33 µg	16 ± 3	20 ± 6	47 ± 4	127 ± 20	509 ± 32
		100 µg	14 ± 2	18 ± 2	31 ± 7	129 ± 11	511 ± 47
		333 µg	13 ± 2[PM]	23 ± 3[PM]	35 ± 2[PM]	93 ± 10[PM]	617 ± 20[PM]
		1000 µg	13 ± 2[PM]	16 ± 3[PM]	33 ± 7[PM]	105 ± 16[PM]	502 ± 13[PM]
		2500 µg	15 ± 1[PM]	7 ± 2[PM]	37 ± 4[PM]	105 ± 7[PM]	466 ± 44[PM]
		5000 µg	13 ± 4[PM]	6 ± 2[PM]	26 ± 4[PM]	115 ± 11[PM]	440 ± 39[PM]
	2-AA	2.5 µg	586 ± 1	288 ± 4	2839 ± 546	2895 ± 159	
		10.0 µg					1515 ± 401

D. DEFICIENCIES

No deficiencies were identified.

III. CONCLUSIONS

Under the experimental conditions of this study, BCS-CN88460 did not induce gene mutation by either base pair changes or frameshift in the Salmonella typhimurium strains used.

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Report:	KCA 5.4.1/02; [redacted]; 2014; M-495533-01-1
Title:	BCS-CN88460, technical: Chromosome aberration test in human Lymphocytes *in vitro*
Report No.:	1614803
Document No.:	M-495533-01-1
Guideline(s):	OECD Guidelines for Testing of Chemicals No. 473 Commission Regulation (EC) No. 440/2008, B10 US EPA (TSCA) OPPTS 870.5375 METI, MHLW, MAFF
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Executive Summary
The test item BCS-CN88460 was dissolved in DMSO and assessed for its potential to induce structural chromosomal aberrations in human lymphocytes *in vitro* in two independent experiments. The following study design was performed:

	Without S9 mix		With S9 mix
	Experiment 1	**Experiment 2**	**Experiments 1 and 2**
Exposure period	4 hours	22 hours	4 hours
Recovery	18 hours		18 hours
Preparation interval	22 hours	22 hours	22 hours

In each experimental group, two parallel cultures were analyzed. Per culture at least 100 metaphases were evaluated for structural chromosomal aberrations, except for the positive control in Experiment 2, in the absence of S9 mix, where only 50 metaphases were evaluated.

The highest applied concentration in the pre-test (4248.0 µg/ml of the test item, approximately 10 mM) was chosen with regard to the molecular weight and the purity (94.2%) of the test item.

Dose selection for the cytogenetic experiment was performed taking into consideration the toxicity data and the precipitation in the test system of the test item.

In the absence and presence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentration.

In Experiment 1, in the absence of S9 mix, one single statistically significant increase in chromosomal aberrations (3.3% aberrant cells, excluding gaps), slightly above the range of the laboratory historical solvent control data (0.0-3.0% aberrant cells, excluding gaps), was observed after treatment with 13.6 µg/mL. No dose dependency was observed. In the presence of S0 mix, no relevant increase was observed.

In Experiment 2, in the absence of S9 mix, one statistically significant increase in chromosomal aberrations (7.0% aberrant cells, excluding gaps), clearly above the range of laboratory historical solvent control data (0.0-2.5% aberrant cells, excluding gaps), was observed after continuous treatment with 6.1 µg/mL. In the presence of S9 mix, one statistically significant increase in chromosomal aberrations (4.5% aberrant cells, excluding gaps), above the range of the laboratory historical solvent control data (0.0-3.5% aberrant cells, excluding gaps) was observed after treatment with 40.0 µg/ml. After treatment with 30.0 µg/mL, one incresae in chromosomal aberrations without statistical significance (3.8% aberrant cells, excluding gaps), but slightly exceeding the range of the laboratory historical solvent control data (0.0-3.5% aberrant cells, excluding gaps) was observed.

No evidence of an incresae in polyploid metaphases was observed after treatment with the test item as compared to the control cultures.

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Appropriate mutagens were used as positive controls. They induced statistically significant increases in the incidence of cells with structural chromosomal aberrations.

In conclusion, it can be stated that under the experimental conditions reported, the test item induced structural chromosomal aberrations in human lymphocytes *in vitro*. Therefore, BCS-CN88460 is considered to be clastogenic in this chromosome aberration test in the absence and presence of S9 mix, whn tested up to cytotoxic and / or precipitating concentrations.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2%
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2015

2. Control materials:	
Negative:	none
Solvent:	Dimethylsulfoxide (DMSO)
Positive:	Without metabolic activation: ethylmethane sulfonate (EMS) With metabolic activation: cyclophosphamide (CPA)

3. Activation: phenobarbital / ß-naphthoflavone-induced rat liver S9, used at a final concentration of 0.75 mg/ml in the culture medium.

4. Test cells: human lymphocytes, prepared from one single non-smoking donor per experiment; for Experiment 1 blood was collected from a 33-year-old female donor and for experiment 2 blood was collected from a 32-year-old male donor.

5. Culture medium: Dulbecco's Modified Eagles Medium / Ham's F12 (DMEM/F12, mixture 1:1), supplemented with 200 mM GlutaMAXTM, penicillin/streptomycin (100 U/mL / 100 µg/mL), the mitogen PHA (3 µg/mL), 10% fetal bovine serum (FBS), 10 mM HEPES, and heparin (125 USP-U/mL)

6. Test compound concentrations used: The concentrations used in the pre-test, Experiment 1, and Experiment 2 are shown in the following table.

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Table 5.4.1/02-1: Concentrations of BCS-CN88460 used in the pre-test, Experiment 1, and Experiment 2 of the *in vitro* chromosome aberrations study using human lymphocytes

	Pre-test		Experiment 1		Experiment 2	
	- S9	+ S9	- S9	+ S9	- S9	+ S9
Exposure period, h	4	4	4	4	22	4
Preparation interval, h	22	22	22	22	22	22
Concentration of BCS-CN88460 µg/mL	9.0	9.0	0.16	2.5	0.4	2.5
	15.8	15.8	0.27	4.5	0.6	5.0
	27.6	27.6	0.47	7.8	1.1	***10.0***
	48.3[P]	48.3[P]	0.83	***13.6***	***2.0***	***20.0***
	84.5[P]	84.5[P]	1.5	***23.9***	***3.5***	***30.0***
	147.8[P]	147.8[P]	2.5	***41.8[P]***	***6.1***	***40.0***
	258.6[P]	258.6[P]	4.5	73.1[P]	10.7	50.0[P]
	452.6[P]	452.6[P]	***7.8***	127.9[P]	18.7	60.0[P]
	792.1[P]	792.1[P]	***13.6***	223.9[P]	32.7[P]	70.0[P]
	1386.1[P]	1386.1[P]	***23.9[P]***	391.8[P]	57.1[P]	80.0[P]
	2425.7[P]	2425.7[P]	41.8[P]	685.7[P]	100.0[P]	100.0[P]
	4245.0[P]	4245.0[P]	73.1[P]	1200.0[P]		500.0[P]

B. TEST PERFORMANCE

1. **In life dates:** 26 February-14 May 2014

2. **Preliminary cytotoxicity assay**
A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment, with 10 concentrations of the test item. The conditions used were identical to those used in the main experiment, described below.

3. **Cytogenetic assay**
All cell cultures were set up in duplicate. Exposure time was 4 hours, both with and without S9 mix. The preparation interval was 22 hours after start of the exposure.

For cultures without S9 mix, at about 48 hours after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm^2 cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10% FBS) containing BCS-CN88460, and the culture medium was then not changed until preparation of the cells.

For cultures with metabolic activation, for cultures without S9 mix, at about 48 hours after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm^2 cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item, and 50 µL of S9 mix per mL of culture medium was added. After 4 hours, the cells were spun down by gentle centrifugation for five minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G", with the washing procedure repeated once. After washing, the cells were resuspended in complete culture medium with 10% FBS and cultured until preparation of the cells.

For cell preparation, the cultures were treated with colcemid at a final concentration of 0.2 µg/mL approximately three hours before the requested harvest time. Cultures were harvested by centrifugation at 22 hours after the beginning of treatment. The supernatant was discarded and the cells were resuspended in hypotonic solution and allowed to stand at 37°C for 20 minutes. After removal of the hypotonic solution by centrifugation at approximately 900x g, the cells were fixed with 3:1 methanol: glacial acetic acid. A small amount of cell suspension was dropped onto clean wet microscope slides, allowed to dry,

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stained with Giemsa, and mounted and covered with a cover slip. All slides were labeled with a computer-generated random code to prevent scorer bias.

At least 100 well-spread metaphases were evaluated per culture for structural aberrations, except for the Experiment 2 positive control without S9 mix, where only 50 metaphases were evaluated. Only metaphases containing at least 46 ± 1 centromeres were included in the analysis. Breaks, fragments, deletions, exchanges, and chromosomal disintegrations were recorded as structural chromosomal aberrations. Gaps were recorded but not included in the calculation of aberration rates.

4. Statistics

Potential statistical significance of the results was determined using the Fisher's exact test ($p < 0.05$).

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

In the preliminary cytotoxicity assay, clear cytotoxicity was observed at the highest evaluated concentrations, as indicated by clearly reduced mitotic indices.

Table 5.4.1/02-2: Cytotoxicity observed in the preliminary cytotoxicity assay

		Mitotic cells, per 1000 cells		% of solvent control	
		- S9	+ S9	- S9	+ S9
Exposure period, h		4	4	4	4
Preparation interval		22	22	22	22
Solvent control		42.6	6.2	100.0	100.0
Concentration of BCS-CN88460, µg/ml	9.0	7.2	4.3	57.1	68.3
	15.8	4.8	4.6	37.7	74.8
	27.6	5.5	3.2	43.7	52.0
	48.4[P]	2.2	1.7	17.5	26.8
	84.5[P]	3.3	2	25.8	35.8
	147.8[P]	0.0	3.8	0.0	61.8
	258.6[P]	0.0	2.7	0.0	43.1
	452.6[P]	0.0	0.0	0.0	0.0
	792.1[P]	0.0	2.8	0.0	44.7
	1386.4[P]	0.0	3.4	0.0	54.5
	2425.7[P]	0.0	0.0	0.0	0.0
	4245.0[P]	0.0	1.7	0.0	27.6

B. CYTOGENETIC ASSAYS

In Experiment 1, in the absence of S9 mix, there was a statistically significant increase in chromosomal aberrations at 13.6 µg/mL. There was no dose dependency. In the presence of S9 mix, there was no relevant increase in the incidence of chromosomal aberrations.

In Experiment 2 in the absence of S9 mix, there was a statistically significant increase in the incidence of chromosomal aberrations at 6.1 µg/mL. In the presence of S9 mix, there was a statistically significant increase in the incidence of chromosomal aberrations at 30 and 40 µg/mL. There was no increase in the incidence of polyploid metaphases in either experiment.

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Table 5.4.1/02-3: Summary of the results of the chromosomal aberration study with BCS-CN88460

Exp.	Exposure, h	S9	Conc., µg/mL	Mitotic index, % control	Incl. gaps	Excl. gaps	Carrying exchanges
1	4	-	Solvent	100.0	0.5	0.5	0.0
			Positive control	76.6	10.5	9.5S	3.5
			7.8	83.1	0.5	0.5	0.0
			13.6	88.8	3.8	3.3S	0.0
			23.9	43.2	3.0	2.5	0.0
	22	+	Solvent	100.0	1.5	1.5	1.0
			Positive control	52.2	15.5	15.5S	2.5
			13.6	117.8	1.5	1.5	0.0
			23.9	88.9	2.0	1.5	0.5
			41.8	53.3	2.5	2.5	0.0
2	22	-	Solvent control	100.0	1.5	1.5	0.0
			Positive control	49.8	65.0	64.0S	12.0
			2.0	84.4	0.5	0.5	0.0
			3.8	92.8	3.5	2.5	0.0
			6.4	45.1	7.0	7.0S	0.0
	4	+	Solvent control	100.0	2.5	1.5	0.0
			Positive control	46.2	25.5	25.0S	3.0
			10.0	79.2	3.0	3.0	0.0
			20.0	66.0	1.5	1.5	0.0
			30.0	57.6	4.0	3.8	0.0
			40.0	35.4	5.8	4.5S	0.0

C. DEFICIENCIES

No deficiencies were identified.

III. CONCLUSIONS

The test item BCS-CN88460 induced structural chromosomal aberrations in human lymphocytes *in vitro*, when tested up to cytotoxic and / or precipitating concentrations.

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Report:	KCA 5.4.1/03; [redacted]; 2014; M-488526-01-1
Title:	BCS-CN88460, technical: Gene mutation assay in Chinese hamster V79 cells *in vitro* (V79 / HPRT)
Report No.:	1614802
Document No.:	M-488526-01-1
Guideline(s):	Ninth Addendum to OECD Guidelines for Testing of Chemicals", February 1998, adopted July 21, 1997, Guideline No. 476: "Commission Regulation (EC) No. 440/2008 B17", dated May 30, 2008; United States Environmental Protection Agency "Health Effects Test Guidelines, OPPTS 870.5300, *In vitro* Mammalian Cell Gene Mutation Test, EPA 712-C-98-221, August 1998; Japanese Guidelines: Kanpoan No. 287 -- EPA Eisei No. 127 -- Ministry of Health & Welfare Heisei 09/10/31 Kikyoku No. 2 -- Ministry of International Trade & Industry; Ministry of Agriculture, Forestry and Fisheries of Japan. MAFF Notification No. 12 Nousan-8147, 24 November 2000
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

The study was performed to investigate the potential of BCS-CN88460 to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster. The study was performed in two independent experiments, using identical experimental procedures. In the first experiment, the treatment period was 4 hours with and without metabolic activation. The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation.

In the pre-experiment, the maximum concentration of 4200 µg/mL was equal to a molar concentration of about 10 mM. The concentration range of the main experiments was limited by cytotoxicity and precipitation. DMSO was used as a solvent.

No substantial or reproducible dose-dependent increase in mutation frequency was observed up to the maximum concentration with and without metabolic activation.

Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies and thus showed the sensitivity of the test system and the activity of the metabolic activation system.

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce gene mutations at the HPRT locus in V79 cells. BCS-CN88460 is considered to be non-mutagenic in this HPRT assay.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2%
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

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2. Control materials:	
Negative:	none
Solvent:	Dimethyl sulfoxide (DMSO)
Positive:	Without metabolic activation: ethylmethane sulfonate (EMS) With metabolic activation: 7,12-dimethylbenz(a)anthracene (DMBA)

3. Activation: phenobarbital/ß-naphthoflavone-induced rat liver S9, used at a final concentration of 0.75 mg/ml in the culture medium

4. Test cells: V79 Chinese hamster lung cells

5. Locus examined: Hypoxanthine-guanine phosphoribosyl transferase (HPRT)

6. Test compound concentrations used: The following table shows the concentrations of BCS-CN88460 used in each experimental condition.

Table 5.4.1/03-1: Concentrations of BCS-CN88460 used in the mammalian cell gene mutation assay

	Experiment 1		Experiment 2	
	- S9	+ S9	- S9	+ S9
Exposure period, h	4	4	24	4
Concentration of BCS-CN88460 µg/mL	2.0	4.0	4.0	4.0
	4.0	**8.0**	**8.0**	**8.0**
	8.0	**16.0**	**16.0**	**16.0**
	16.0	**32.0**	**32.0**	**32.0**
	24.0[P]	**48.0**	**48.0[P]**	**48.0[P]**
	32.0[P]	**64.0[P]**	**64.0[P]**	**64.0[P]**
				96.0[P]
				128.0[P]

B. TEST PERFORMANCE

1. In life dates: 25 February – 6 May 2014

2. Cell treatment

A pre-test was performed in order to determine the concentration range for the mutagenicity experiments, using the same culture conditions and experimental conditions as for the mutagenicity experiments. In this pre-test, the colony-forming ability of approximately 500 single cells (duplicate cultures per concentration level) after treatment with the test item was observed and compared to the controls. Toxicity of the test item was indicated by a reduction of the cloning efficiency.

The pre-experiment was performed in the presence (4 hours treatment) and absence (4 and 24 hours treatment) of metabolic activation. Test item concentrations ranged from 32.8 to 4200 µg/mL. A strong cytotoxic effect occurred at 32.8 µg/mL after 4 hours treatment without metabolic activation, and cytotoxicity was also observed at 32.8 µg/mL and above after 24 hours treatment without metabolic activation. In the presence of metabolic activation, strong toxic effects were noted at 65.6 µg/mL and above. Precipitation occurred at 32.8 µg/mL and above without metabolic activation, and at 131.3 µg/mL and above with metabolic activation.

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For seeding and treatment of the cell cultures, the complete culture medium was minimum essential medium (MEM) containing Hank's salts, 10% FBS (except during 4-hour treatment), neomycin (5 µg/mL), and amphotericin B (1%). For the selection of mutant cells, the complete medium was supplemented with 11 µg/mL 6-thioguanine. All cultures were incubated at 37°C in a humidified atmosphere with 1.5% CO2.

Two to three days after subcultivation, stock cultures were trypsinized at 37°C for 5 minutes, then the trypsinization was stopped by adding complete cell culture medium with 10% FBS and a single cell suspension was prepared. Approximately 1.5 x 10^6 (single culture) and 5 x 10^2 cells (in duplicate) were seeded in plastic culture flasks. The cells were grown for 24 hours prior to treatment.

At treatment, the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After four hours, the cells were washed twice with "saline G" and the cultures were given complete medium. In the second experiment, the cells were exposed to the test item for 24 hours in complete medium, supplemented with 10% FBS, in the absence of metabolic activation.

Three or four days after treatment, 1.5 x 10^6 cells per experimental point were sub-cultivated in 175 cm^2 flasks containing 30 mL medium. Following the expression time of seven days, five 80 cm^2 cell culture flasks were seeded with about 3-5 x 10^5 cells each in medium containing 6-thioguanine. Two additional 25 cm^2 flasks were seeded with approximation 500 cells each in non-selective medium to determine the viability. Cultures were incubated at 37°C in a humidified atmosphere with 1.5% CO2 for about 8 days, and colonies were then stained with 10% methylene blue in 0.01% KOH solution. Stained colonies with more than 50 cells were counted.

3. Statistics

A linear regression (least squares) was performed to assess a possible dose-dependent increase in mutant frequencies. The number of mutant colonies obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05%. However, both biological and statistical significance were considered together.

4. Evaluation Criteria

The gene mutation assay is considered acceptable if it meets the following criteria:

- The number of mutant colonies per 10^6 cells found in the solvent controls falls within the laboratory historical control data range.
- The positive control substances produce a significant increase in mutant colony frequencies.
- The cloning efficiency II (absolute value) of the solvent controls exceeds 50%.

A test item is classified as positive (mutagenic) if it induces either a concentration-related increase in the mutant frequency, or a reproducible and positive response at one of the test points. A test item producing neither a concentration-related increase of the mutant frequency nor a reproducible positive response at any of the test points, is considered non-mutagenic in this system.

A test item is classified as mutagenic if it reproducibly induces a mutation frequency that is three times above the spontaneous mutation frequency in at least one of the concentrations in the experiment, or if there is a reproducible concentration-related increase of the mutation frequency even when a three-fold increase in mutation frequency is not observed.

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II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

Test item concentrations ranged from 32.8 to 4200 µg/mL. A strong cytotoxic effect occurred at 32.8 µg/mL after 4 hours treatment without metabolic activation, and cytotoxicity was also observed at 32.8 µg/mL and above after 24 hours treatment without metabolic activation. In the presence of metabolic activation, strong toxic effects were noted at 65.6 µg/mL and above. Precipitation occurred at 32.8 µg/mL and above without metabolic activation, and at 131.3 µg/mL and above with metabolic activation.

Table 5.4.1/03-2: Relative cloning efficiency in cells cultured with BCS-CN88460 at varying concentrations

		Experimental conditions		
		- S9	+ S9	- S9
	Exposure time, h	**4**	**4**	**24**
Concentration of BCS-CN88460 µg/mL	Solvent control	100.0	100.0	100.0
	32.8	15.2	96.4	42.2^{P}
	65.6	0.0^{P}	2.8	8.7^{P}
	131.3	0.0^{P}	1.0^{P}	1.7^{P}
	262.5	0.0^{P}	0.5^{P}	0.0^{P}
	525.0	0.0^{P}	0.0^{P}	0.0^{P}
	1050.0	0.0^{P}	0.0^{P}	0.0^{P}
	2100.0	0.0^{P}	5.5^{P}	0.0^{P}
	4200.0	0.0^{P}	48.7^{P}	0.3^{P}

B. MUTATION ASSAYS

The first experiment was conducted with an exposure period of 4 hours, both with and without S9 metabolic activation. In the incubations without metabolic activation, precipitation of the test item was noted at the end of the treatment period at concentrations of 24.0 µg/mL and above. With metabolic activation, precipitation was noted at 64.0 µg/mL and above. Cytotoxicity was observed in the absence of metabolic activation at concentrations of 16.0 µg/mL and above.

In the second experiment, with an exposure period of 4 hours in the presence of S9 metabolic activation and 24 hours without S9 metabolic activation, cytotoxicity was observed at 32.0 µg/mL and above without and at 48.0 µg/mL with metabolic activation.

No relevant and reproducible increase in mutant colony numbers per 10^6 cells was observed in the main experiments up to the maximum concentration tested.

The threshold of three times the mutation frequency of the corresponding solvent control was reached or exceeded in the first experiment at 4.0 and 24.0 µg/mL without metabolic activation (culture 1), at 32.0 µg/mL without metabolic activation (culture II), and in the first experiment at 32.0 and 64.0 µg/mL with metabolic activation (culture II). However, all of these effects were judged as irrelevant fluctuations based on relatively low solvent controls. The absolute mutation frequency remained well within the historical range of solvent controls, and none of these increases was reproduced in the parallel cultures under identical conditions. In the second experiment, another isolated increase was noted at the maximum concentration of 128 µg/mL in culture II with metabolic activation. Both the three-fold threshold and the range of historical solvent controls were exceeded. This increase was judged as a biologically irrelevant precipitation artifact as it occurred far into the precipitating concentration range and was not reproduced in the parallel culture under identical experimental conditions.

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A linear regression analysis (least squares) was performed to assess a possible dose-dependent increase in mutation frequencies. A significant dose dependent trend of the mutation frequency, indicated by a probability value < 0.05, was solely determined in the second culture of the second experiment with metabolic activation. This trend was however judged irrelevant as it was based on the precipitation artefact discussed above.

In both experiments of this study, both with and without the S9 mix, the range of the solvent controls was from 3.5 up to 28.3 mutants per 10^6 cells; the range of the groups treated with the test item was from 3.3 up to 48.5 mutants per 10^6 cells.

EMS at 150 µg/mL and DMBA at 1.1 µg/mL were used as positive controls and showed the expected increase in induced mutant colonies.

Table 5.4.1/03-3: Summary of the results of the HPRT-locus mammalian gene mutation *in vitro* assay with BCS-CN88460

			Culture I			Culture II		
Treatment	**Conc., µg/mL**	**S9**	**CE II, %**	**Mutant colonies / 10^6 cells**	**Induction factor**	**CE II, %**	**Mutant colonies / 10^6 cells**	**Induction factor**
DMSO		-	100.0	3.8	1.0	100.0	3.5	1.0
EMS	150.0		103.4	138.0	36.6	96.0	94.5	26.7
BCS-CN88460	4.0		98.8	5.4	1.4	99.0	5.0	1.4
	8.0		100.0	9.4	2.5	96.6	3.3	0.9
	16.0		102.0	6.6	1.8	98.5	6.8	1.9
	24.0P		102.9	27.7	7.3	97.5	3.8	1.1
	32.0P		100.4	10.6	2.8	95.7	11.2	3.2
DMSO		+	100.0	6.6	1.0	100.0	6.9	1.0
DMBA	1.1		93.9	177.3	26.8	97.8	221.9	32.0
BCS-CN88460	8.0		96.7	7.2	1.1	101.2	5.4	0.8
	16.0		94.6	4.9	0.7	100.9	14.8	2.1
	32.0		97.0	5.3	0.8	99.3	22.9	3.3
	48.0		97.8	12.5	1.9	99.7	7.4	1.1
	64.0P		96.8	4.7	0.7	102.6	20.5	3.0
DMSO		-	100.0	28.3	1.0	100.0	12.6	1.0
EMS	150.0		99.1	429.1	15.2	100.7	421.3	33.5
BCS-CN88460	8.0		101.5	4.8	0.2	104.9	13.2	1.0
	16.0		101.5	9.3	0.3	101.2	12.9	1.0
	32.0P		98.9	6.9	0.2	97.5	10.8	0.9
	48.0P		104.6	12.7	0.4	100.6	6.8	0.5
	64.0P		101.2	30.0	1.1	101.3	16.5	1.3
DMSO		+	100.0	22.5	1.0	100.0	15.3	1.0
DMBA	1.1		97.3	115.9	5.2	105.2	147.5	9.6
BCS-CN88460	8.0		94.8	5.9	0.3	103.2	15.8	1.0
	16.0		96.4	25.4	1.1	105.0	20.2	1.3
	32.0		65.7	23.0	1.0	105.8	25.2	1.6
	48.0P		99.9	15.8	0.7	102.7	10.8	0.7
	64.0P		94.6	4.4	0.2	105.9	24.7	1.6
	128.0P		100.2	4.2	0.2	102.9	48.5	3.2

C. DEFICIENCIES

No deficiencies were noted.

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III. CONCLUSIONS

Under the experimental conditions described, the test item did not induce gene mutations at the HPRT locus in V79 cells. Thus, BCS-CN88460 is non-mutagenic in this mammalian gene mutation assay.

CA 5.4.2 *In vivo* studies in somatic cells

Report:	KCA 5.4.2/01; [redacted]; 2014; M-485866-01-1
Title:	BCS-CN88460, technical - Micronucleus assay in bone marrow cells of the mouse
Report No.:	1614804
Document No.:	M-485866-01-1
Guideline(s):	OECD No. 474, adopted July 21, 1997 EC No. 440/2008 B12", dated May 30, 2008 EPA OPPTS 870.5395, EPA 712-C-98-226, August, 1998
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

This study was performed to investigate the potential of BCS-CN88460 to induce micronuclei in polychromatic erythrocytes in the bone marrow of the mouse.

The test item was dissolved in DMSO / PEG 400 (3/7), which was also used as vehicle control. Both test item and vehicle control were administered twice orally at an interval of 24 hours. The volume administered twice orally was 10 ml/kg bw/day (total volume of applications was 20 mg/kg bw). At 48 hours after the first administration of the test item (24 hours after the last treatment) the bone marrow cells were collected for micronuclei analysis.

Seven males per test group were evaluated for the occurrence of micronuclei. Per animal, 2000 polychromatic erythrocytes (PCEs) were scored for micronuclei.

To determine whether the treatment had had a cytotoxic effect, the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and reported as the number of PCEs per 2000 erythrocytes.

As estimated by pre-experiments, 2000 mg BCS-CN88460/kg bw administered twice orally (2 x 2000 mg/kg bw) was suitable as the highest treatment dose. Thus, the dose levels of 500, 1000, and 2000 mg/kg bw twice orally were investigated in the mutagenicity experiment.

After treatment with the test item, the number of PCEs was not substantially decreased as compared to the mean value of PCEs of the vehicle control, thus indicating that BS-CN88460 did not exert any cytotoxic effects in the bone marrow.

In comparison to the corresponding vehicle control, there was no biologically relevant or statistically significant increase in the frequency of the detected micronuclei at any preparation interval and any dose level.

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

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In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the micronucleus test with bone marrow cells of the mouse. Therefore, BCS-CN88460 is considered to be non-mutagenic in this micronucleus assay.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2%
CAS #	1255734-28-1
Stability of test compound:	Until 15 July 2014

2. Control materials:	
Negative:	none
Solvent:	30% dimethyl sulphoxide / 70% polyethylene glycol (DMSO / PEG 400 (3/7)
Positive:	cyclophosphamide

3. Test animals:	
Species:	mouse
Strain:	NMRI
Age:	8-12 weeks
Weight at dosing:	36.0 ± 1.7g at first application; 36.4 ± 1.7g at second application
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Pelleted standard diet, ad libitum
Water:	Tap water ad libitum
Housing:	Single housing in Makrolon cages with wire mesh top
Environmental conditions:	
Temperature:	22 ± 3°C
Humidity:	Approximately 23-65%
Air changes:	Not specified
Photoperiod:	12 hours light, 12 hours dark

4. Test compound concentrations used: Pre-test: 2 doses of 2000 mg/kg bw at 24-hour intervals; main study: 2 doses of 500, 1000, or 2000 mg/kg bw at 24-hour intervals

B. TEST PERFORMANCE

1. In life dates: 26 February 2014-11 March 2014

2. Treatment and sampling times

In the pre-test, groups of two animals per sex were administered BCS-CN88460 by oral gavage twice at intervals of 24 hours, and were examined for symptoms of acute toxicity at intervals of 0-1, 2-4, 5-6, and 24 hours after each administration of the test item.

In the definitive study, groups of seven male mice per dose level were administered BCS-CN88460 by oral gavage twice at intervals of 24 hours, at doses of 500, 1000, or 2000 mg/kg bw. Bone marrow was then collected at 24 hours after the second treatment.

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3. **Tissues and cells examined**
Animals were sacrificed using carbon dioxide asphyxiation followed by exsanguination. Femurs were removed, the epiphyses were cut off, and the marrow was flushed out with fetal bovine serum. The resulting cell suspension was centrifuged at 390x g for 10 minutes, the supernatant was discarded, the cell pellet was re-suspended, and a small drop of this suspension was spread on a slide, air-dried, stained with May-Gruenwald/Giemsa, and mounted. At least one slide was made from each bone marrow sample.

4. **Details of slide analysis**
Evaluation of the slides was performed using 100x oil immersion microscope objectives. Per animal, 2000 polychromatic erythrocytes were analyzed for micronuclei. The ratio between polychromatic and normochromatic erythrocytes was determined in the same sample, and expressed at polychromatic erythrocytes per 2000 erythrocytes.

5. **Evaluation criteria**
The study is considered valid if at least five animals per group could be evaluated, if the PCE to erythrocyte ratio was not less than 20% of the ratio in the negative control animals, and if the positive control showed a statistically significant and biologically relevant increase in micronucleated PCEs compared to the vehicle control.

A test item is considered genotoxic if it induces either a dose-related increase or a clear increase in the number of micronucleated PCEs in a single dose group above the historical control range for the solvent controls.

6. **Statistical methods**
The non-parametric Mann-Whitney test was used to evaluate the results.

II. RESULTS AND DISCUSSION

A. RANGE-FINDING TEST

In the range-finding study, after two administrations of BCS-CN88460 by oral gavage at 2000 mg/kg bw, both males and females showed similar clinical signs, with no substantial difference in toxicity between the sexes.

B. MICRONUCLEUS ASSAY

1. **Toxicity**
Clinical signs of toxicity were observed in a dose-related manner at all doses.

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Table 5.4.2/01-1: Clinical signs of toxicity observed in male mice after gavage administration of a first and second dose of BCS-CN88460

			Time after treatment, in hours			
Dose	**Observation**	**Application**	**0-1**	**2-4**	**5-6**	**24**
500 mg/kg bw	Reduction of spontaneous activity	1	3	0	0	0
		2	0	1	0	0
	Ruffled fur	1	4	4	4	3
		2	4	4	4	3
1000 mg/kg bw	Reduction of spontaneous activity	1	5	5	0	0
		2	0	0	0	0
	Abdominal position	1	1	0	0	0
		2	0	0	0	0
	Eyelid closure	1	2	0	0	0
		2	0	0	0	0
	Ruffled fur	1	5	5	6	4
		2	5	5	7	4
	Apathy	1	4	0	0	0
		2	0	0	0	0
2000 mg/kg bw	Reduction of spontaneous activity	1	6	4	0	0
		2	0	0	0	0
	Abdominal position	1	1	0	0	0
		2	0	0	0	0
	Eyelid closure	1	2	0	0	0
		2	0	0	0	0
	Ruffled fur	1	7	7	7	5
		2	7	7	7	5
	Apathy	1	1	0	0	0
		2	0	0	0	0
	Excitement	1	0	0	0	0
		2	1	0	0	0

2. PCE ratio

There was no effect of treatment with BCS-CN88460 on the ratio of PCEs to normochromatic erythrocytes, indicating that BCS-CN88460 was not cytotoxic to the bone marrow.

3. Micronucleated polychromatic erythrocytes (MPCEs)

There was no effect of treatment with BCS-CN88460 on the incidence of micronucleated PCEs at any dose. In contrast, the administration of cyclophosphamide at 40 mg/kg bw as a positive control markedly increased the incidence of micronucleated PCEs, indicating that the assay was valid.

Table 5.4.2/01-2: Incidence of micronucleated PCEs in male mice administered BCS-CN88460 at up to 2000 mg/kg bw

	BCS-CN88460, dietary concentration in ppm				Cyclophosphamide, 40 mg/kg bw
	0	500	1000	2000	
PCEs with micronuclei, %	0.093	0.093	0.086	0.129	1.850
Range	1-3	0-4	1-2	0-4	22-68
PCE/2000 erythrocytes	1242	1202	1174	1202	1116
Significance		n.t.	n.t.	-	+
P value		-	-	0.1906	0.003

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C. DEFICIENCIES

1. Under the 1997 OECD test guideline 474, only 2000 polychromatic erythrocytes were required to be examined per animal, in contrast to the 4000 currently required. Given that there was no relevant increase in the incidence of micronucleated PCEs at any dose up to 2000 mg/kg bw, it is unlikely that the results would have differed if 4000 PCEs were examined per animal as is required by the 2016 OECD test guideline 474.

2. This study was conducted in 2014 under the 1997 version of the relevant OECD test guideline 474, which did not require assessment of plasma levels of the test substance nor was any other means of demonstrating exposure of bone marrow required. The study was therefore in compliance with the OECD test guideline in force at the time of study conduct.

Since the study was conducted, however, new requirements have been imposed specifying that *in vivo* genotoxicity studies must demonstrate that the test item has reached the target tissue. In its 2017 Scientific Opinion (doi: 10.2903/j.efsa.2017.5113), EFSA specified that several lines of evidence could be considered in determining whether or not the bone marrow had been exposed to the test item. These lines of evidence, and the data available for each of them with BCS-CN88460, are listed below.

- Test substance (and/or metabolites) detected in the bone marrow in a toxicokinetic study:
  - The study listed under data point 5.1.1/03 shows that radioactivity is observed in the bone marrow of rats following oral gavage administration of radiolabelled BCS-CN88460. This line of evidence can therefore be used to demonstrate exposure of the target tissue to BCS-CN88460 and its metabolites.
- Systemic toxicity observed in the bone marrow micronucleus test:
  - Clinical signs of toxicity including abdominal position, ruffled fur, reduction in spontaneous activity, apathy, and/or excitement were observed in all treated groups in a dose-related manner. These clinical signs indicate systemic toxicity of BCS-CN88460 after oral gavage administration in the mouse. This line of evidence can therefore be used to demonstrate exposure of the target tissue to BCS-CN88460 and its metabolites.
- Systemic toxicity observed in toxicity studies:
  - In the mouse, for example in the 28-day study (see data point 5.3.1/02), indications of systemic toxicity including increased ASAT and ALAT and decreased bilirubin, increased liver weight, and increases in the incidence of centrilobular hepatocellular hypertrophy, hepatocellular necrotic foci, and focal hepatocellular single cell necrosis were observed after dietary administration of BCS-CN88460 to male and female mice. As the primary target organ of BCS-CN88460 is the liver, these findings indicate systemic toxicity of the test item. This line of evidence can therefore be used to demonstrate exposure of the target tissue to BCS-CN88460 and its metabolites.
  - In the rat, in addition to findings in the liver, male rats in the 28-day and 90-day study showed an increased incidence of kidney findings (e.g., cellular casts in the urine, increased incidence of hyaline droplets) with increasing dietary concentrations of BCS-CN88460. These findings in a target organ other than the liver indicate that the test item or its metabolites were absorbed and entered the systemic circulation. As the bone marrow is a highly-perfused tissue, substances which enter the systemic circulation and cause systemic toxicity beyond the liver must be assumed to have reached the bone marrow. This line of evidence can therefore be used to demonstrate exposure of the target tissue to BCS-CN88460 and its metabolites.
- Test substance (and/or metabolites) detected systemically in a toxicokinetic study: Both the test item and a number of metabolites were detected systemically after oral

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gavage administration of the radiolabelled test compound to male and female rats (see data point 5.1). This line of evidence can therefore be used to demonstrate exposure of the target tissue to BCS-CN88460 and its metabolites.

- Test substance detected systemically in a specific blood / plasma analysis:
  - In the mouse chronic study, plasma samples from the 12-month and 18-month time-points showed relatively high concentrations of two metabolites of BCS-CN88460, as well as low concentrations of the test item itself (see data point 5.5/02). As the bone marrow is a highly-perfused tissue, detection of the test item or its metabolites in blood or plasma indicates that they will have reached the bone marrow. This line of evidence can therefore be used to demonstrate exposure of the target tissue to BCS-CN88460 and its metabolites.
- Toxicity to the bone marrow observed in the mammalian erythrocyte micronucleus test: as there were no changes in the PCE/NCE ratio in the mouse *in vivo* micronucleus study, this line of evidence does not apply.
- Toxicity to the bone marrow observed in toxicity studies: As there were no indications of any effects on the bone marrow in any repeat-dose study in any experimental species, this line of evidence does not apply.

The above summary shows that several of the lines of evidence proposed in the EFSA 2017 Scientific Opinion "Clarification of some aspects related to genotoxicity assessment" for demonstrating that a test item has reached the target tissue in an *in vitro* study can be used in the case of BCS-CN88460. Thus, it can be accepted that BCS-CN88460 reached the bone marrow in the present *in vivo* mouse micronucleus study and this study is acceptable under the afore-mentioned EFSA Scientific Opinion.

III. CONCLUSIONS

The test item BCS-CN88460 did not induce any increase in the incidence of micronucleated PCEs in the bone marrow of male mice administered BCS-CN88460 at up to the limit dose of 2000 mg/kg bw. Thus, it can be concluded that under the conditions of this assay, BCS-CN88460 is not clastogenic in the *in vivo* mouse micronucleus assay.

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CA 5.4.3 *In vivo* studies in germ cells

As no indication of an effect on fertility was observed in either males or females in the 2-generation reproduction study (M-612750-02-1 referenced in this Summary MCA Sections under Point 5.6.1/01) nor was there any visible effect on histopathology of the seminiferous tubules which might have indicated an effect on male gametes, no *in vivo* genotoxicity studies in germ cells were conducted.

CA 5.5 Long-term toxicity and carcinogenicity

Table 5.5-1: Summary of long-term toxicity and carcinogenicity studies with [Substance]

Study	NOAEL	LOAEL	Effects
Rat 2-year chronic study Dietary study 0, 30, 150, 450 (males) or 800 (females) ppm [redacted]; 2018; M-612739-02-1	Males: 450 ppm (18.6 mg/kg bw/day) Females: 800 ppm (46.6 mg/kg bw/day)	Males: > 450 ppm Females: > 800 ppm	Thyroid colloid alteration, pigment accumulation
Mouse 18-month chronic study Dietary study 0, 50, 250, 1250 ppm [redacted]; 2017; M-593645-01-1	250 ppm (Males: 29.0 mg/kg bw/day Females: 38.1 mg/kg bw/day)	1250 ppm (147 / 190 mg/kg bw/d males / females)	Decreased body weight / body weight gain, increased liver weight, histopathological findings in the liver

Based on knowledge from other compounds of this chemical class as well as from short-term studies in the rat with BCS-CN88460, the liver and thyroid had been identified as key target organs in the rat. Additionally, the kidney was a clear target organ in the male rat, with a trend towards accelerated or exacerbated chronic progressive nephropathy. A worsening of chronic progressive nephropathy in the male rat can lead to increased mortality in a chronic study, making it difficult to have sufficient animals at the end of the study to judge the chronic and oncogenic effects of the test item. Dose selection for the chronic study in the rat required a balancing of the liver and kidney effects, which had been shown to be greater in the female, with the need to avoid excessive mortality in the males due to chronic progressive nephropathy.

These considerations led to the selection of 0, 30, and 150 ppm for both sexes, with a high dose of 450 ppm in the males and 800 ppm in the females. Over the course of the study, these dietary concentrations provided systemic doses of 0, 1.2, 6.3, and 18.6 mg/kg bw/day in the males and 0, 1.7, 8.5, and 46.6 mg/kg bw/day in the females. In the males, there were no adverse effects noted throughout the study. Total bilirubin was slightly decreased in males at 450 ppm, and the incidence of diffuse pigmentation of the thyroid follicular cells was slightly increased at the top dose. These are not adverse findings; decreased bilirubin is a non-adverse marker of exposure to chemicals such as BCS-CN88460, while follicular cell pigmentation is a degenerative finding indicating a slight and prolonged stimulation of the thyroid gland by the test item. In females, at 800 ppm there was a slight decrease on body weight and body weight gain on several occasions during the study, although overall body weight gain was only decreased by 4% at the end of the study. Food consumption was also slightly reduced, but without any biological significance. Absolute and relative liver weights were increased at 800 ppm, but without any accompanying histopathological changes. There was an increase in females at 800 ppm of both thyroid colloid alteration and thyroid follicular cell pigmentation, however these findings are considered to be non-adverse, degenerative findings

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indicating a slight and prolonged stimulation of the thyroid gland by the test item. No neoplastic effects were seen in either males or females at any dose. The NOEL for the study was 150 ppm (6.3 mg/kg bw/day in males, 8.5 mg/kg bw/day in females), while the NOAEL was greater than the highest dose tested, 450 ppm (18.6 mg/kg bw/day) in males and 800 ppm (46.6 mg/kg bw/day) in females.

The highest consumer exposure to BCS-CN88460 or its residues is 0.00063 mg/kg bw/day. Although biologically significant toxic effects were not demonstrated in the 2-year rat chronic study, the NOEL of 6.3 mg/kg bw/day represents a 10,000-fold margin of exposure over the highest predicted consumer exposure. This clearly demonstrates that a safe risk assessment can be performed even in the absence of significant toxicity in this study.

In the mouse, the identification of the liver as a key organ in short-term studies was used to guide dose selection for the mouse chronic study. Based on increases in liver weight and on the changes in hepatocellular vacuolation seen at 1000 ppm, dietary concentrations of 0, 50, 250, and 1250 pm were selected for the mouse 18-month study. These provided systemic doses of 0, 5.9, 29.0, and 147 mg/kg bw/day in males and 0, 7.8, 38.1, and 190 mg/kg bw/day in females. Overall body weight and body weight gain were significantly reduced in both males (by 11%) and females (by 13%). Absolute and relative liver weights were increased at both 12 and 18 months. At 18 months, there was also an increase in absolute and relative kidney weights in males, and in adrenal weights in both sexes. The only treatment-related findings in either sex were an increase in the incidence of multinucleated hepatocytes in males (12 and 18 months), bile duct hyperplasia in females (12 and 18 months), focal single cell necrosis in the liver of males only (18 months only), and hepatocellular necrotic focus(i) in females only (18 months only), and a slight decrease in severity of diffuse hepatocellular vacuolation (18 months, females only). In the kidney, male mice showed increased incidence of hyaline casts and focal dilation of the tubules in the medulla, as well as an increase in the severity of focal tubular basophilia. No neoplastic effects were seen in either males or females, however based on the decrease in overall body weight gain and on the alterations in liver weight and histopathology, sufficient toxicity was observed at 1250 ppm. The NOAEL for this study was 250 ppm (29.0 mg/kg bw/day in males and 38.1 mg/kg bw/day in females).

Report:	KCA 5.5/01; [redacted]; 2018; M-612739-02-1
Title:	BCS-CN88460: Chronic toxicity and carcinogenicity study in the wistar rat by dietary administration
Report No.:	SA 13266
Document No.:	M-612739-02-1
Guideline(s):	OECD 453 (September, 2009) EEC Directive 88/302/EEC, Method B.33 (November, 1987) US EPA OCSPP guideline number 870.4300 MAFF in Japan, notification 12 Nousan n° 8147 (November, 2000)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

The objective of this study was to investigate the chronic toxicity and oncogenic potential of BCS CN88460 in Wistar rats following continuous dietary treatment at 30, 150 and 450 ppm for male rats and 30, 150 and 800 ppm for female rats over a 24-month period, according to the following design:

Groups of 70 male and 70 female rats were fed diet containing 0, 30, 150 and 450 ppm BCS CN88460 (batch number 2013-006492, a beige solid, 94.2% w/w purity) for male rats for at least 103 weeks and 0, 30, 150 and 800 ppm BCS-CN88460 for female rats for at least 105 weeks. Ten males and ten females from each group were allocated to the chronic (12-month) phase and were necropsied after 52 weeks of treatment. The remaining 60 animals/sex/group were allocated to the carcinogenicity

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(24-month) phase of the study, continued treatment until final sacrifice of the study after at least 103 and 105 weeks for male and female rats respectively.

Mortality and clinical signs were checked daily. Detailed physical examinations including palpation for masses were performed at least weekly throughout the study. Body weight was recorded weekly for the first 13 weeks, then approximately every 4 weeks thereafter. Food consumption was recorded twice weekly for the first 6 weeks of the study, then approximately weekly up to Week 13, then every 4 weeks thereafter. Ophthalmology examinations were performed on all animals during acclimatization on animals of control and group 4 after approximately one year of treatment, and on all surviving animals at two years of treatment. On study weeks 16, 52, 102 and 103 (study days 108, 358, 714 and 718), a blood sample was collected from the sublingual vein of the first five suitable animals from each treated group for test item analysis. Hematology and clinical chemistry determinations and urinalysis were performed during months 3-4, 6-7, 12, 18 and 24 on selected animals. At the scheduled chronic and carcinogenicity phase sacrifice, selected organs were weighed and designated tissues sampled and examined microscopically.

The mean achieved dose levels of BCS-CN88460 received by the animals over the 12-month period were approximately 1.416, 7.17 and 21.3 mg/kg/day in males and 1.968, 9.08 and 52.1 mg/kg/day in females, corresponding to the dietary levels of 30, 150 and 450 ppm for male rats and 30, 150 and 800 ppm for female rats.

The mean achieved dose levels of BCS-CN88460 received by the animals over the 24-month period were approximately 1.237, 6.27 and 18.6 mg/kg/day in males and 1.746, 8.54 and 46.6 mg/kg/day in females, corresponding to the dietary levels of 30, 150 and 450 ppm for male rats and 30, 150 and 800 ppm for female rats.

No treatment-related effects were noted on the mortality rate in males and females at any dose level. No treatment-related cause of death or macroscopic or microscopic changes were established for the animals allocated to the chronic phase (12 months) or the carcinogenicity phase (24 months) of the study which died or were humanely sacrificed before the end of these two phases of treatment.

In both sexes, there were no treatment-related effects on the following parameters, at any dose-level tested: ophthalmic changes, hematology and urinalysis parameters. In addition, no treatment-related effects on mean terminal body weights were observed at either the 12-month interim or 24-month terminal sacrifice. No macroscopic or microscopic changes related to administration of the test item were observed in animals allocated to the 12-month interim sacrifice phase and no macroscopic changes that could be attributable to treatment were observed at the 24-month sacrifice.

No treatment-related neoplastic changes were observed in either sex at any dose level.

The bioanalytical examination showed relatively low levels of BCS-CN88460 mean plasma concentrations for both sexes throughout the study. In males at 450 ppm, the observed concentration was slightly greater than the limit of quantification after 2 years of treatment.
In females, at 150 and 800 ppm, the plasma concentration of the test item was slightly increased in a generally dose-related manner observed after one year of treatment. At 800 ppm, these plasma concentrations were decreased after 24-month of treatment.

A dose-related increase in BCS-CX99799 and BCS-CX99798 mean plasma concentrations was observed for both sexes after 12 and 24 months of treatment. In all cases the mean plasma concentrations for the two main metabolites were higher in females compared to males.

After 12 months of treatment, the bioanalytical results showed higher concentrations of BCS CX99799 compared to the plasma concentrations of BCS-CX99798 for both sexes (except for female rats exposed to 800 ppm for which the BCS-CX99798 concentrations were higher). After 24 months of

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treatment, the highest concentrations were detected for BCS-CX99798 in both sexes compared to the BCS-CX99799 plasma concentrations.

At 450 ppm (equating to 18.6 mg/kg bw/day) in males / 800 ppm (equating to 46.6 mg/kg/day) in females
Over the 2-year duration of the study, the mortality rate of both sexes was markedly lower than in the corresponding control groups (56.7% versus 71.7% for males and 51.7% versus 55.9% for females, adjusted for censored animals).

In females, a higher incidence of hair loss was observed ($p \leq 0.05$) in comparison to controls.

In females, a slight reduction of 4% in mean body weight was observed at study days 316 and 344 (statistically significant $p \leq 0.05$ or $p \leq 0.01$). Mean cumulative body weight gain statistically significant in some occasions ($p \leq 0.05$ or $p \leq 0.01$) was reduced from Study Day 50 until the end of the study, resulting in a decrease in overall mean cumulative body weight gain of 4% at Study Day 729 (not statistically significant).

Over the study period, treatment-related changes in food consumption were observed in female rats. Mean food consumption was slightly reduced during the first week of treatment by approximately 4% ($p \leq 0.05$). Thereafter, mean food consumption was reduced from Study Day 204 until the end of the study and was statistically significant at several occasions ($p \leq 0.05$ or $p \leq 0.01$).

Clinical chemistry determination revealed slightly lower mean total bilirubin concentrations in females at each period. Total bilirubin concentration decrease was considered not to be adverse as there were no related microscopic changes and as it does not represent any functional impairment in the test organism. It is, however, a consistent indicator of administration of a compound acting through activation of the CAR and / or PXR receptors.

After 12-month of treatment, mean absolute and relative liver weights were significantly higher in females only (+11% to +20%, $p \leq 0.05$ or $p \leq 0.01$) when compared with controls.
At the end of the carcinogenicity phase, liver-to-body weight ratio in females was significantly higher (+12%, $p \leq 0.01$) when compared with controls.

After 24-month of treatment, microscopic pathology examination revealed treatment-related non neoplastic findings in the follicular cells of the thyroid gland in male and female rats. In both sexes, higher incidence and severity of colloid alteration was noted compared to the control group and appeared statistically significant in females only ($p \leq 0.001$). In males, a significantly higher incidence of diffuse pigmentation of the follicular cells ($p \leq 0.05$) was observed. These degenerative findings can be associated with the slight and prolonged thyroid gland stimulation and therefore were not considered to be adverse.
In male rats, the thyroid gland was affected by a significantly higher incidence of diffuse C-cell hyperplasia ($p \leq 0.05$). This finding commonly seen in aging Wistar rats was not associated with any signs of hyperparathyroidism or hypercalcemia and therefore was not considered to be related to administration of the test item.
In the adrenal gland, a significantly higher incidence of focal medullary hyperplasia was observed in the males ($p \leq 0.05$). However no clear dose response was observed in the intermediate dose groups and severity was not affected. Focal medullary hyperplasia is a common finding in aging male Wistar rats, and although the incidence appeared significantly increased, the p-value remained high; therefore this finding was not considered to be related to administration of the test item.

At 150 ppm (6.27 mg/kg bw/day in males and 8.54 mg/kg bw/day in females)
In male rats, a lower mortality rate was noted over the 2-year duration of the study, compared to the controls (61.7% versus 71.7%, adjusted for censored animals).

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At the 24-month terminal sacrifice, a significantly higher incidence of diffuse C-cell hyperplasia in the thyroid gland was observed in the male rats ($p \leq 0.05$). For the reasons already mentioned for animals exposed to the high dose level, this finding commonly seen in aging Wistar rats was not considered to be related to administration of the test item.

At 30 ppm (1.24 mg/kg bw/day in males and 1.75 mg/kg bw/day in females)
Apart from a lower mortality in male rats over the 2-year duration of the study compared to the control group (63.3% versus 71.7%, adjusted for censored animals), no treatment-related changes were noted throughout the study in either sex for other parameters evaluated.

In conclusion, dietary administration of BCS-CN88460 over a 24-month period to the Wistar rat, at dose levels up to 450 ppm for male rats and 800 ppm for female rats did not induce carcinogenic effects. A dose level of 150 ppm (equivalent to 6.27 mg/kg bw/day in males and 8.54 mg/kg bw/day for females) was considered to be a No Observed Effect Level (NOEL) in both sexes over a 24-month period of dietary administration. The No Observed Adverse Effect Level (NOAEL) of dietary administration of BCS-CN88460 over a 24-month period in the Wistar rat was > 450 ppm (18.6 mg/kg body weight/day) in the males and > 800 ppm (46.6 mg/kg body weight/day) in the females.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% (w/w)
CAS #	1255734-28-1
Stability of test compound:	Recertified several times during the conduct of the study

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	rat
Strain:	Wistar Rj:WI (IOPS HAN)
Age:	6 weeks old
Weight at dosing:	182-241g for males; 140-190g for females
Source:	[redacted]
Acclimation period:	13 days
Diet:	A04CPI-10 from [redacted], [redacted], France, ad libitum
Water:	Filtered and softened tap water from the municipal water supply, ad libitum
Housing:	By sex in groups of 5; until 16 November, 2015, housed in suspended stainless steel and wire mesh cages; from 17 November 2015, suspended polycarbonate and wire mesh cages.
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 per hour
Photoperiod:	12 hours dark / 12 hours light

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B. STUDY DESIGN

1. In life dates: 5 February 2014-26 February 2016

2. Dose level selection

The doses used in this study were selected on the basis of the 90-day study ([redacted]; 2017; M-487478-02-1), in which BCS-CN88460 was administered to male and female Wistar rats at dietary concentrations of 0, 100, 300, and 1000 ppm, equivalent to approximately 0, 6.34, 18.4, and 63.5 mg/kg bw/day in males, and 0, 7.92, 21.9, and 80.9 mg/kg bw/day in females.

- At 1000 ppm
  - Mean absolute body weight gain was reduced by 12% in males and 8% in females relative to controls, while mean absolute body weight was reduced by 7% in males and unchanged relative to controls in females.
  - Mean total bilirubin was decreased in both males and females, and total cholesterol was slightly increased in females only. Although decreased total bilirubin is not an adverse effect, it can be considered an indication of liver enzyme activity.
  - Relative liver weight was increased in males, and both absolute and relative liver weights were increased in females. Periportal to panlobular hepatocellular hypertrophy was observed in females, but not in males.
  - Relative thyroid weight was increased in males, but no effect on thyroid weight was observed in females. However, an increased incidence of follicular cell hypertrophy and colloid alteration was observed in both male and female rats.
  - Cellular casts in the urine were observed in 5 of 10 males.
  - Relative kidney weight was increased in males, although this observation is considered related to the slightly lower mean terminal body weight. There was an increase in the incidence and severity of hyaline droplets in proximal tubules and of bilateral basophilic tubules, as well as granular casts in some animals.
- At 300 ppm
  - No effect on body weight or body weight gain in either sex.
  - Mean total bilirubin was decreased in females only.
  - Cellular casts were observed in the urine of one male, although as this was within the historical data it was considered to be non-adverse. In the kidney, there was a slightly higher incidence and severity of hyaline droplets in the proximal tubules, however this observation was considered not to be adverse as it was also seen at the same severity in some control males.
  - No effects on organ weights in either males or females.
- At 100 ppm:
  - The only treatment-related change noted was a decrease in total bilirubin concentrations in females.

During dose selection for the chronic study, it was considered that extended administration of high dietary concentrations of BCS-CN88460 would exacerbate the kidney findings in males, increasing the mortality related to chronic progressive nephropathy and risking the validity of the study. Based on the urinalysis and histopathologic findings at 300 and 1000 ppm after 90 days, it was considered that a dose of 450 ppm would induce significant but not excessive toxicity in males without inducing excessive mortality, and this was selected as the high dose in males.

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The increased absolute and relative liver weights in females at 1000 ppm in the 90-day study, along with the increase in hepatocellular hypertrophy, were considered to indicate a significant effect on the liver, a known target organ for BCS-CN88460 and other members of its class. The increase in thyroid follicular hypertrophy and colloid alteration at this dose are again known effects of BCS-CN88460 and other similar compounds. These effects on known target organs were used to establish a top dose in females at 800 ppm, where it was expected that organ weight, histopathological findings, and possibly tumor incidence would be increased.

Other doses of 150 and 30 ppm were selected with a view to ensuring roughly equivalent dose spacing, as well as to having an expected No Observable (Adverse) Effect Level at 30 ppm and a dose with some toxicity at the mid dose of 150 ppm.

Thus, the concentrations used in this 2-year chronic study in the male and female rat were 0, 30, 150, and 450 ppm in the males and 0, 30, 150, and 800 ppm in females.

3. Animal assignment

All animals were weighed on days 3 and 10 of the acclimatization phase, and subjected to a detailed physical and ophthalmological examination once during acclimatization. On the day of randomization, one day before the start of test item administration, all animals were weighed. On the day of randomization, animals were allocated to dose groups using a computerized randomization procedure (Pristima, version 6.3.2. build 17, Xybion Corp.) that ensured a similar body weight distribution among groups for each sex.

Ten animals per sex were assigned to each dietary concentration including control for an interim sacrifice group, and 60 animals per sex were assigned to each dietary concentration including control for the terminal sacrifice group.

4. Diet preparation and analysis

The test item was incorporated into the diet at concentrations of 30, 150, and 450 ppm for male treatment groups and 30, 150, and 800 ppm for female treatment groups. The formulations were prepared to cover the dietary requirements for four weeks per formulation, apart from the last formulation which was used until the end of the study. Diet formulations were stored at ambient temperature when not in use.

The stability of the test item has previously been demonstrated at concentrations of 60 and 10000 ppm over a 100-day storage period at room temperature. In addition, the stability of the test item was verified on the first batch of the first and second formulations at 30 pm, for a time which covered the period of storage and use on this study. The stability of BCS-CN88460 was checked on single samples that were stored frozen for 91 days and then kept at room temperature for ten days, as well as on samples that were help for 101 days at room temperature.

Sixteen diet formulations were prepared during the study at 150, 450, and 800 ppm. For the 30 ppm diet, seventeen formulations were prepared, as one batch had to be replaced for technical reasons.

The homogeneity of the test item in the diet was verified from the first batches at all concentrations on the first and second formulations, and for 30 and 800 ppm of the seventh and thirteenth formulations. Additionally, the homogeneity of the seventh formulation was checked at 450 ppm. The mean values obtained from the homogeneity analyses were taken as the measured concentration. Finally, the concentration was checked at all dose levels on the first, fourth, seventh, tenth, and sixteenth formulations.

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The homogeneity and concentration analyses showed that both parameters were 85-109% of nominal concentrations. As these were within the in-house acceptability range of 85-115%, the formulations were considered acceptable for use.

5. Statistics

For body weight change parameters, terminal body weight, absolute and relative organ weight, specific hematology parameters, quantitative urinalysis parameters and clinical chemistry parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight and food consumption parameters, and specific hematology parameters, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.
If one or more group variances were equal to 0, means were compared using non-parametric procedures.

A survival analysis was conducted on the carcinogenicity phase. Adjusted survival rates were estimated using Kaplan-Meier estimation procedures, calculated separately for each sex and treatment group. Mortalities which were the result of animals killed following accidents (accidental trauma, animals which died during anesthesia) or at scheduled sacrifices were considered to be censored observations. Statistical differences in survival rates between treated and control groups, and dose-relative trends in survival, were assessed using Log-rank trend test. Pairwise comparison was assessed using Tarone tests with

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Scheffe adjustment for multiple comparisons. Probability values were two-sided for Log-rank test and one-sided for pairwise comparisons.

For neoplastic and non-neoplastic findings, all findings were assessed with a poly-k test (with k=3) taking into account time to death and presence of the finding: firstly, a trend poly-k test was performed regarding all doses. Pairwise comparisons were assessed using poly-k test with Sidak adjustment. Probability values were two-sided for trend poly-k test and one-sided for pairwise comparisons poly-k. Findings were analyzed after 24 months treatment (unscheduled sacrifices, scheduled sacrifice, and combined unscheduled and scheduled sacrifices). All tissues missing, autolytic, non-readable, or inadequate were not used in the analysis.

For selected clinical signs and for ophthalmological and macroscopic observations, in order to test the hypothesis that the treated group incidence of selected clinical signs noted at physical examination and of macroscopic findings (scheduled sacrifice, and combined unscheduled and scheduled sacrifices after one and two years) were not higher than the incidences of the control group, these findings were analyzed using Cochran-Armitage test. Treated groups were then compared using Fisher's exact test with Sidak adjustment where applicable.

Probability values presented were two-sided for Cochran-Armitage trend test and one-sided for pairwise comparisons. When the finding incidence of the treated group was lower than the finding incidence of the control group, no statistical test was performed.

C. METHODS

1. Observations

Animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily, and the nature, onset, severity, duration, and recovery from clinical signs were recorded. Cages and cage trays were inspected daily for evidence of ill health such as blood or loose feces. In addition, detailed physical examinations including palpation for masses were performed at least weekly. The onset, location, and dimension of any masses were recorded.

2. Body weight

Each animal was weighed at least weekly during the acclimatization period then weekly for the first 13 weeks of the study, then approximately every four weeks thereafter, and prior to necropsy.

3. Food consumption and compound intake

The weight of food supplied, and of that remaining at the end of each food consumption period, was recorded for each cage. Food consumption was recorded twice weekly during the first six weeks of treatment, then weekly up to week 13, and once approximately every four weeks thereafter. Food consumption and body weight data were used to calculate the mean achieved dosage intake in mg/kg bw/day.

4. Ophthalmoscopic examination

During the acclimatization phase, all animals were examined by indirect ophthalmoscopy. During the treatment period, funduscopic (indirect ophthalmoscopy) and biomicroscopic (slit lamp) examinations were performed on all surviving animals after approximately 12 and 24 months of treatment with BCS-CN88460. Each eye was examined by direct ophthalmoscopy in the first instance, and then after instillation of an atropinic agent, each eye was re-examined by means of a slit lamp and an indirect ophthalmoscope.

5. Hematology and clinical chemistry

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Analyses for hematology and clinical chemistry were performed on all surviving animals of the 12-month interim sacrifice group in weeks 13/15, 26/27/28, and 52/53, and on the first ten surviving rats of the terminal sacrifice groups in weeks 13/15, 26/27/28, 52/53, 76, and 102. Animals were diet-fasted overnight and anesthetized by isoflurane inhalation for blood collection by puncture of the retro-orbital venous plexus. Blood was collected onto EDTA for hematology, clot activator for clinical chemistry, and sodium citrate for coagulation. At terminal sacrifice, blood smears were prepared for all animals not sampled for hematology. Standard hematology and clinical chemistry parameters were examined.

6. **Urinalysis**
Animals were diet- and water-fasted during the overnight (approximately 16 hours) urine collection period. Urine samples were collected from all surviving animals of the 12-month interim sacrifice group in weeks 14, 25, and 51, and on the first ten surviving rats of the terminal sacrifice groups in weeks 14, 26-27, 51, 78, and 101. Standard urinalysis parameters were examined.

7. **Test item analysis**
After approximately three months of treatment, as well as at the end of 12 months and during the month before final sacrifice, a blood sample was collected from the sublingual vein of five suitable animals per sex from the groups receiving the test item. Animals were not diet-fasted overnight prior to blood collection. Based on a preliminary blood kinetic analysis study, blood was collected at approximately 8am. Prior to sample collection, animals were anesthetized by isoflurane inhalation. Blood was collected into heparinized vials, and plasma was prepared by centrifugation then stored in the dark at approximately -20°C until analysis.

8. **Sacrifice and pathology**
On study days 371 to 373 for the 12-month interim sacrifice, and on study days 715 to 739 for the carcinogenicity phase, all surviving animals in the respective groups were sacrificed by exsanguination under deep anesthesia ensured by isoflurane inhalation. An approximately equal number of animals randomly distributed among all groups were sampled on each day at the interim sacrifice. Animals were diet-fasted overnight prior to sacrifice.

All animals, including those either found dead or killed for humane reasons, were necropsied. The necropsy included the examination of external surfaces, all orifices, and all major organs, tissues, and body cavities. All significant macroscopic abnormalities including masses and their regional lymph nodes where possible were recorded, sampled, and examined microscopically.

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Table 5.5/01-1: Organs and tissues which were sampled and/or weighed at necropsy

	Digestive system		**Cardiovasc. / Hemat.**		**Neurologic**
X	Tongue	X	Aorta, thoracic*	XX	Brain (3 sections)*+
X	Submandibular (salivary) gland	XX	Heart*+		
		X	Bone marrow, sternum*	X	Sciatic nerve*
X	Esophagus*	X	Lymph node, mesenteric*	X	Spinal cord (cervical, thoracic, lumbar)*
X	Stomach*				
X	Duodenum*	X	Lymph node, submaxillary	X	Eyes (retina)*
X	Jejunum*			X	Optic nerves*
X	Ileum*	XX	Spleen*+		
X	Cecum*	XX	Thymus		**Glandular**
X	Colon*			XX	Pituitary gland*
X	Rectum*		**Urogenital**	XX	Adrenal gland*+
XX	Liver*+	XX	Kidney*+	X	Parathyroid gland*
X	Pancreas*	X	Urinary bladder*	XX	Thyroid gland* (weighed with parathyroid gland)+
		XX	Testis*+		
	Respiratory	XX	Epididymis*+		
X	Trachea*	XX	Prostate gland*	^	Lacrymal exorbital gland
X	Lung*	X	Seminal vesicle*		
^	Pharynx*	XX	Ovary*+		**Other**
^	Larynx*	XX	Uterus (with cervix)*+	X	Bone (sternum)
^	Nasal cavities	X	Mammary gland*	X	Skeletal muscle
		X	Vagina	X	Skin*
				X	All gross lesions and masses
				X	Articular surface (femorotibial joint)

* required for chronic studies based on US EPA guideline 870.4300
\+ organ weights required for rodent studies
X tissues were collected for histological examinations
XX organs were weighed fresh at scheduled sacrifice only, with paired organs weighed together, and collected for histological examination
^ organs were preserved for possible micropathologic examination only

Two femoral bone marrow smears were prepared from sacrificed animals (except on weekends and public holidays), one of which was stained with May-Gruenwald Giemsa and the other was stored unstained. These smears were not examined as no relevant changes were observed in hematology or bone marrow histology.

Tissue samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye and optic nerve, Harderian gland, epididymis, and testis, which were fixed in Davidson's fixative.

Tissues (except for exorbital lacrymal gland, larynx and pharynx, and nasal cavities) were embedded in paraffin wax, and histological sections were prepared and stained with hematoxylin and eosin.

For the 12-month sacrifice animals, histological examinations were conducted on all organs and tissues from animals sacrificed or dying during the treatment period and from animals in the control and high dose groups, the liver, lung, and thyroid from animals of the intermediate dose groups, and gross abnormalities from all animals.

For the carcinogenicity phase, histopathological examinations were performed on all organs and tissues including gross abnormalities from all animals in all groups including decedents.

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II. RESULTS AND DISCUSSION

A. MORTALITY

In the chronic phase of the study, there was no increase in mortality in either males or females. In the carcinogenicity phase of the study, mortality rate among treated males in all groups was lower than in the corresponding controls. Tthe male section of the study was terminated during week 102 because male mortality in the control group was approaching 75%, the threshold for potential early termination of a study (OECD Test Guideline 453, OECD Guidance Document 116). In females, mortality was slightly decreased at 800 ppm compared to the control group. Thus, BCS-CN88460 did not increase mortality in either males or females after either chronic or lifetime dietary administration.

Table 5.5/01-2: Mortality rate in male and female rats administered BCS-CN88460 via the diet for up to two years

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	30	150	450	0	30	150	800
Initial N, day 0	70	70	70	70	70	70	70	70
Killed for humane reasons	2	4	2	2	2	-	5	
Found dead	1	-	2	1	-	-	1	-
Died during anesthesia	-	-	-	-	1	-	-	
Accidental trauma	1	-	-	-	-	-	-	
Total deaths	4	-	6	3	3	2	1	5
Initial N, day 366	60	60	60	60	60	60	60	60
Killed for humane reasons	13	16	22	17	29	24	28	25
Found dead	30	22	15	17	4	12	12	6
Died during anesthesia	-	-	-	-	1	-	-	-
Accidental trauma	-	-	-	-	-	-	-	-
Total deaths	43	38	37	34	33	36	40	31

B. CLINICAL OBSERVATIONS

There were no treatment-related clinical signs observed in males at any dose level at any time during the study. In females in both the chronic and the carcinogenicity phases of the study, there was an increased incidence of hair loss noted at the top dose of 800 ppm.

C. BODY WEIGHT

Body weight was not affected in males at any dose during any phase of the study. In females, there was no effect on body weight or body weight gain at either 30 or 150 ppm. At 800 ppm, body weight was slightly reduced during the second half of the study. Body weight gain was reduced from study day 50 at this dose level, and was occasionally statistically significant. At the end of the study in females at 800 ppm mean overall body weight gain was reduced by 4% relative to controls (not statistically significant). Thus, administration of BCS-CN88460 to male and female rats in the diet did not affect either body weight or body weight gain in a biologically meaningful manner.

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Table 5.5/01-3: Mean body weight and body weight gain in male and female rats administered BCS-CN88460 in the diet for up to two years

		BCS-CN88460, dietary concentration in ppm							
		Males				Females			
Parameter	**Day**	**0**	**30**	**150**	**450**	**0**	**30**	**150**	**800**
Body weight, g	1	214.1	213.6	213.6	214.5	165.0	165.6	165.9	165.7
	29	391.7	391.4	389.5	387.7	241.5	243.5	245.4	239.6
	91	533.7	537.2	536.2	530.6	295.1	295.8	301.1	291.1
	316	670.4	673.7	667.7	664.7	352.7	354.1	358.6	337.1*
	344	681.6	681.2	679.9	671.7	359.5	357.4	365.6	339.5**
	540	700.8	722.8	714.2	708.6	417.5	416.1	439.1	394.3
	708	665.4	695.8	670.6	699.0	446.1	470.2	456.2	435.4
	729					454.5	468.4	464.9	442.7
Body weight gain, g	1-29	177.6	177.8	175.9	173.2	76.6	77.9	79.5	74.0
	1-91	319.8	323.6	322.4	316.1	130.0	130.2	135.2	125.4
	1-344	467.6	467.7	466.9	457.1	194.0	191.6	199.6	174.0**
	1-540	488.6	508.6	501.2	495.8	253.6	251.7	273.3	231.0*
	1-708	456.3	479.8	460.5	488.8				
	1-729					392.0	304.3	303.3	280.5

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

D. FOOD AND WATER CONSUMPTION

There was no effect on food consumption in males at any dose in this study at any time point. In females, at 800 ppm, food consumption was slightly decreased (approximately 4% decreased) during the first week of the study and from study day 204 until the end of the study. These decreases were occasionally statistically significant. However, in light of the similar reductions in body weight and body weight gain, never greater than 4% in the top dose in females, these occasional reductions in food consumption were not biologically significant.

E. ACHIEVED INTAKE

Compound intake is shown in Table 5.5/01-4 for various periods of the study.

Table 5.5/01-4: Mean intake of BCS-CN88460, in mg/kg bw/day in male and female rats treated via the diet

	BCS-CN88460, dietary concentration in ppm					
	Males			Females		
Weeks	**30**	**150**	**450**	**30**	**150**	**800**
1-13	1969	9.88	29.3	2.484	12.14	66.2
1-52	1.416	7.17	21.3	1.968	9.68	52.1
1-102/105	1.237	6.27	18.6	1.746	8.54	46.6

F. OPHTHALMOSCOPIC EXAMINATIONS

There were no ophthalmological findings at any point of the study, in either males or females, which were related to treatment.

G. HEMATOLOGY, CLINICAL CHEMISTRY, AND URINALYSIS

1. Hematology

There were no changes in hematological parameters in either males or females which were related to dietary administration of BCS-CN88460.

2. Clinical Chemistry

Total bilirubin concentration was decreased at the top dose in both males (450 ppm) and females (800 ppm) at all measurements, and was generally statistically significant. Although this decrease in total bilirubin does not represent a toxic effect, it serves as an indication that the compound was working through the CAR-PXR mode of action.

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Table 5.5/01-5: Mean total bilirubin, in µmol/L in male and female rats administered BCS-CN88460 via the diet for up to two years

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
Month	**0**	**30**	**150**	**450**	**0**	**30**	**150**	**800**
3/4	1.35	1.21	1.34	0.64**	1.75	1.66	1.64	0.66**
7	1.35	1.21	1.34	1.02*	2.04	1.94	1.87	1.00***
12	1.75	1.48	2.07	1.28*	2.87	2.87	2.80	1.29**
18	1.99	1.65	1.94	1.27	2.80	2.33	1.86	1.27***
24	2.03	1.63	1.97	1.42*	2.05	1.95	1.80	1.01**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3. Urinalysis

There were no treatment-related changes in urinalysis parameters in either males or females during the study.

4. Bioanalytical examinations

Plasma samples were analyzed for concentrations of BCS-CN88460 at 3 to 4 months of dietary administration of the compound, and for BCS-CN88460 as well as the metabolites BCS-CX99799 and BCS-CX99798 at 12 and 24 months of the study.

In the control group, concentrations for the parent compound and its metabolites were close to or below the limit of quantification of 0.01 mg/L in both males and females. The trace amounts detected in the plasma of control animals is considered attributable to negligible cross contamination in the animal facility (e.g. dust generated during changing of feeders.

The parent compound was detected at relatively low levels in both males and females throughout the study. Dose-related increases of the metabolites were generally observed in both sexes, although plasma concentrations were greater in females than in males.

Table 5.5/01-6: Mean plasma concentrations of BCS-CN88460, BCS-CX99799, and BCS-CX99798 in male and female rats administered BCS-CN88460 via the diet for up to two years

	BCS-CN88460, dietary concentration in ppm					
	Males			Females		
3/4 months	**30**	**150**	**450**	**30**	**150**	**450**
BCS-CN88460	< LOQ	0.010	0.019	< LOQ	< 0.012	0.015
12 months						
BCS-CN88460	0.01	< 0.015	< 0.015	< 0.01	0.014	0.068
BCS-CX99799	0.074	0.320	0.523	0.155	0.423	0.813
BCS-CX99798	0.020	0.123	0.377	0.015	0.143	1.278
24 months						
BCS-CN88460	< 0.010	< 0.01	0.032	< 0.01	0.017	0.027
BCS-CX99799	< 0.015	0.069	0.310	0.012	0.083	1.230
BCS-CX99798	0.111	0.540	0.895	0.121	0.682	1.470

H. SACRIFICE AND PATHOLOGY

I. Terminal body weight and organ weight

At both the one-year and the two-year timepoints, there was no effect in either males or females on terminal body weights, but both absolute and relative liver weights were increased in females in the 800 ppm group.

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Table 5.5/01-7: Mean terminal body weight and absolute and relative liver weight in male and female rats administered BCS-CN88460 via the diet for up to two years

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	**30**	**150**	**450**	**0**	**30**	**150**	**800**
12m, N ex.	8	10	9	10	10	8	10	10
Terminal body wt, g	684.0	674.7	659.4	654.0	340.3	366.8	342.1	362.8
Liver wt, g	13.1	13.4	12.9	13.4	7.2	7.8	7.6	8.0*
Liver wt / body wt, %	1.9	2.0	2.0	2.1	2.1	2.1	2.2	2.3*
Liver wt / brain wt, %	558.5	583.9	564.5	572.6	340.7	382.0	365.3	407.1**
24m, N ex.	17	22	23	26	26	24	20	29
Terminal body wt, g	626.9	661.8	639.1	663.1	430.3	440.8	430.1	417.5
Liver wt, g	12.3	13.2	12.5	13.2	9.5	9.8	9.6	10.3
Liver wt / body wt, %	2.0	2.0	2.0	2.0	2.2	2.2	2.2	2.5**
Liver wt / brain wt, %	533.7	561.5	537.2	569.5	444.9	459.2	447.0	489.4

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

2. Macroscopic findings

There were no macroscopic findings which were related to treatment in either males or females during the study.

3. Microscopic findings

After twelve months, there were no findings which were considered related to administration of the test item.

At two years, there were no neoplastic findings which were related to administration of the test compound. Non-neoplastic, treatment-related findings were observed in the thyroid follicular cells in both males and females at the respective top doses. Both the incidence and the severity of colloid alteration was noted in both males and females, and the incidence of diffuse pigmentation of the follicular cells was increased in males as well.

Both of these are degenerative findings which are associated with the slight, prolonged thyroid gland stimulation due to the CAR-PXR mode of action. Neither colloid alteration nor pigmentation of the follicular cells is considered to be adverse.

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Table 5.5/01-8: Incidence and severity of treatment-related histopathological findings in male and female rats administered BCS-CN88460

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	30	150	450	0	30	150	800
N examined	59	59	59	59	60	60	60	59
Colloid alteration								
Minimal	21	16	21	21	10	6	18	23
Slight	6	8	8	16	1	0	1	5
Moderate	0	0	0	1	0	0	0	0
Total	27	24	29	38	11	6	19	28***
Pigmentation, follicular cells, diffuse								
Minimal	2	3	6	11	2	0	0	5
Total	2	3	6	11*	2	0	0	5

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

I. DEFICIENCIES

The study as described was compliant with the technical requirements of the relevant guidelines. None the less, dose selection was deficient as no adverse findings were noted.

III. CONCLUSIONS

Dietary administration of BCS-CN88460 over a 24-month period to the Wistar rat, at dose levels up to 450 ppm for male rats and 800 ppm for female rats, did not induce carcinogenic effects. The dose level of 150 ppm (6.27 mg/kg bw/day in males, 8.54 mg/kg bw/day in females) was considered to be a No Observed Effect Level (NOEL) in both sexes. The No Observed Adverse Effect Level (NOAEL) for dietary administration of BCS-CN88460 over a 24-month period in the Wistar rat was > 450 ppm (18.6 mg/kg bw/day) in the males and > 800 ppm (46.6 mg/kg bw/day) in females.

Report: KCA 5.5/02; [redacted]; 2017; M-593645-01-1
Title: BCS-CN88460 - Carcinogenicity study in the c57bl/6j mouse by dietary administration
Report No.: SA 13273
Document No.: M-593645-01-1
Guideline(s): OECD 451 (September, 2009)
EEC Directive 88/302/EEC, Method B.32 (November, 1987)
US EPA OCSPP Guideline number 870.4200
MAFF in Japan, notification 12 Nousan n°8147 (November, 2000)
Guideline deviation(s): none
GLP/GEP: yes

Executive Summary

The objective of this study was to investigate the oncogenic potential of BCS-CN88460 in the C57BL/6J mouse following continuous dietary treatment for 18 months. In addition, an interim sacrifice was performed after 52 weeks of treatment to assess chronic toxicity.

Groups of 60 male and 60 female C57BL6J mice were fed diet containing 0, 50, 250, or 1250 ppm of BCS-CN88460 for 52 weeks. After 52 weeks, 10 males and 10 females from each group allocated to the chronic phase of the study were necropsied at the scheduled interim sacrifice. The remaining 50 animals per sex per group, allocated to the carcinogenicity phase of the study, continued treatment until the scheduled final sacrifice of the study after at least 78 weeks of treatment (equating to a mean intake of BCS-CN88460 over 18 months of 0, 5.9, 29.0, and 147 mg/kg bw/day in males and 0, 7.8, 38.1, and 190 mg/kg bw/day in females, respectively). Mortality and clinical signs were checked daily. Additionally, detailed physical examinations including palpation for masses were performed

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weekly throughout treatment. Body weight and food consumption were measured for the first 13 weeks of the study, then monthly thereafter. Hematology determinations were performed at 13 and 19 months from designated animals. When possible, blood smears were prepared from moribund animals just before sacrifice. A blood sample was also collected on selected animals for bioanalytical examinations after 3 months and one year and at the end of the study. All animals were subjected to necropsy, with selected organs weighed at scheduled interim and final sacrifice. Designated tissues were fixed and examined microscopically.

Up to the highest dietary concentration tested of 1250 ppm, there were no treatment-related effects observed at physical examination, on mean food consumption and hematology assessments throughout the study, or on the latency period to or incidence of tumors in either sex.

At bioanalytical examination BCS-CN88460 was found below or slightly above the LOQ throughout the study in all treated groups. A dose-related increase in the mean plasma concentrations of the two major test item metabolites BCS-CX99798 and BCS-CX99799 was observed with similar levels observed throughout the study and higher mean values in females compared to males, especially for BCS-CX99799.

At 1250 ppm (equating to 147 and 190 mg/kg bw/day in males and females respectively)
There was a marginal increase in the mortality incidence in females only compared to controls. The body weight parameters were slightly affected in both sexes. The mean body weight was lower than controls in both sexes by up to 6%. Overall, the mean cumulative body weight gain between day 1 and day 540 (weeks 1-78) was 11% lower than controls in males and 13% lower than controls in females.

Chronic phase necropsy: at the 1-year interim sacrifice, mean terminal body weights were unaffected by treatment in either sex. At necropsy in both males and females, the mean absolute and relative liver weights were statistically significantly higher when compared with controls. At the macroscopic examination, no treatment-related changes were noted in either sex. At microscopic examination, in the liver, a higher incidence of multinucleated hepatocytes was noted in males, and a higher incidence of bile duct hyperplasia was observed in females when compared with controls.

Carcinogenicity phase necropsy: at the 18-month terminal sacrifice of the carcinogenicity phase animals, mean terminal body weights were decreased by 6% in both sexes. At necropsy, in males the mean absolute and relative liver weights were statistically significantly higher than in controls. The mean absolute and relative kidney weights were statistically significantly higher in males when compared to controls. At the macroscopic observation, no treatment-related changes were noted in either sex. At the microscopic examination, treatment-related findings were noted in the liver, and in males only in the kidneys.

In males, a higher incidence and severity of multinucleated hepatocytes (43/50 males) and single cell necrosis (10/50 animals) were observed in the liver when compared to controls. In the kidney, a higher incidence of hyaline casts (46/50 animals), a higher incidence of tubule dilation in the medulla (4/50 animals), and a slightly higher severity of focal tubule basophilia were observed when compared to controls.

In females, in the liver, a higher incidence and severity of diffuse bile duct hyperplasia (25/50 animals), a higher incidence of hepatocellular necrotic foci (24/50 animals), and a lower incidence and severity of diffuse hepatocellular vacuolation were observed compared to controls.

In addition, a slightly higher incidence and / or severity of amyloid deposition was observed in liver, submaxillary lymph node, kidney in females only, adrenal gland, and thyroid gland.

<u>At 250 ppm (equating to 29.0 and 38.1 mg/kg bw/day in males and females, respectively)</u>

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No treatment-related effects were observed at this dietary concentration of BCS-CN88460.

At 50 ppm (equating to 5.9 and 7.8 mg/kg bw/day in males and females, respectively)
No treatment-related effects were observed at this dietary concentration of BCS-CN88460.

In conclusion, dietary administration of BCS-CN88460 over an 18-month period to the C57 BL/6J mouse up to a dietary concentration of 1250 ppm (equivalent to 147 mg/kg bw/day in males and 190 mg/kg bw/day in females) did not induce carcinogenic effects.

The No Observed Adverse Effect Level (NOAEL) over an 18-month period of dietary administration of BCS-CN88460 to the C57BL/6J mouse was considered to be 250 ppm (equivalent to 29.0 mg/kg bw/day in males and 38.1 mg/kg bw/day in females).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% (w/w)
CAS #	1255734-28-1
Stability of test compound:	Until 28 April 2016

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	mouse
Strain:	C57BL/6J
Age:	6 weeks of age
Weight at dosing:	18.5-22.3g for males; 15.0-18.9g for females
Source:	[redacted]
Acclimation period:	14 days
Diet:	A04CP1-10 from [redacted], [redacted], France, ad libitum
Water:	Filtered and softened tap water from the municipal water supply, ad libitum
Housing:	By sex in groups of three from arrival until day 7 of the acclimatization phase, then individually; suspended cages of stainless steel and wire mesh
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15/hour
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN

1. In life dates: 26 February 2014-30 September 2015

2. Dose level selection

The dose levels used in this study were selected based on the results from a 90-day dietary study in the mouse ([redacted]; 2013; M-472773-01-1; see data point 5.3.2/02), in which BCS-CN88460 was added to the diet at concentrations of 0, 100, 300, and 1000 ppm

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(equating to 0, 17.0, 51.0, and 168 mg/kg bw/day in males and 0, 19.5, 59.8, and 207 mg/kg bw/day in females).

At the highest dose of 1000 ppm, absolute and relative liver weights were increased in both sexes, and total bilirubin was decreased. At microscopic examination, there was an increased incidence of diffuse hepatocellular vacuolation. Liver weight was also increased in males at the mid dose of 300 ppm, although there were no findings at histopathological examination. Based on these results, the concentrations of 0, 50, 250, and 1250 ppm were selected for this study.

3. Animal assignment

All animals were weighed on days 3, 8 and 13 of the acclimatization phase, and were subjected to a detailed physical examination during that phase. On the day of randomization, animals were allocated to dose groups using a computerized randomization procedure (Pristima, version 6.3.2 build 17, Xybion Corp., and from 11 September 2015 version 7.0.0 build 22, Xybion Corp.) that ensured a similar body weight distribution for each sex. Selected animals were in a weight range from 18.5 to 22.3g for males, and 15.0 to 18.9g for the females at the start of treatment.

Ten male and ten female mice per group were assigned to the 52 week interim sacrifice group, and 50 male and 50 female mice per group were assigned to the final sacrifice at 78 weeks.

4. Diet preparation and analysis

The test item was incorporated into the diet to provide the required dietary concentrations of 50, 250, or 1250 ppm. The test item formulations were prepared for approximately 8 weeks for the first 10 formulations, and one week for the last formulation. When not in use, the diet formulations at 250 and 1250 ppm were stored at ambient temperatures, while the 50 ppm formulation was kept frozen at approximately -18°C.

The stability of the test item was demonstrated in a previous study, and was verified at 60 and 10000 ppm for up to 100 days when kept at ambient temperature. In addition, the stability of the test item was checked at 30 ppm for a time period which covers the period of storage and usage of the current study. The test item was found to be stable at 30 ppm when kept frozen for 91 days followed by 10 days at room temperature.

Eleven formulations were prepared during the study at each concentration. The homogeneity of the test item was verified at 50 and 1250 ppm on the first, seventh, and eleventh formulations. The mean value obtained from the homogeneity check was taken as the measured concentration.

During the study, the concentration at each dietary level was verified prior to administration for the first, third, seventh, ninth, and eleventh formulations.

The concentration analyses showed 88-108% of nominal concentrations. As these were within the in-house target range of 85-115% of nominal concentrations, the formulations were considered to be acceptable for use on the current study.

Homogeneity analyses were generally at 82-111% of nominal concentrations, with the exception of one value at 80% (seventh formulation at 50 ppm). As these were within the in-house target range of 85-115% of nominal concentrations, the formulations were considered to be acceptable for use on the current study.

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5. Statistics

For body weight change parameters, terminal body weight, absolute and relative organ weight, and specific hematology parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight and food consumption parameters, and specific hematology parameters, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.
If one or more group variances were equal to 0, means were compared using non-parametric procedures.

A survival analysis was conducted on the carcinogenicity phase. Adjusted survival rates were estimated using Kaplan-Meier estimation procedures, calculated separately for each sex and treatment group. Mortalities which were the result of animals killed following accidents (accidental trauma, animals which died during anesthesia) or at scheduled sacrifices were considered to be censored observations. Statistical differences in survival rates between treated and control groups, and dose-relative trends in survival, were assessed using Log-rank trend test. Pairwise comparison was assessed using Tarone tests with Scheffe adjustment for multiple comparisons. Probability values were two-sided for Log-rank test and one-sided for pairwise comparisons.

For neoplastic and non-neoplastic findings, all findings were assessed with a poly-k test (with k=3) taking into account time to death and presence of the finding: firstly, a trend poly-k test was performed regarding all doses. Pairwise comparisons were assessed using poly-k test with Sidak adjustment. Probability values were two-sided for trend poly-k test

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and one-sided for pairwise comparisons poly-k. Findings were analyzed after 18 months treatment (unscheduled sacrifices, scheduled sacrifice, and combined unscheduled and scheduled sacrifices). All tissues missing, autolytic, non-readable, or inadequate were not used in the analysis.

For selected clinical signs and for ophthalmological and macroscopic observations, in order to test the hypothesis that the treated group incidence of selected clinical signs noted at physical examination and of macroscopic findings (scheduled sacrifice, and combined unscheduled and scheduled sacrifices after 12 and 18 months) were not higher than the incidences of the control group, these findings were analyzed using Cochran-Armitage trend test. Treated groups were then compared using Fisher's exact test with Sidak adjustment where applicable.

C. METHODS

1. Observations

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 each day throughout the study. Additional detailed physical examinations including palpation for masses were performed at least weekly throughout the study. The nature, onset, severity, duration, and reversibility of clinical signs, as well as the onset, location, dimension, appearance, progression, and duration of masses was recorded.

2. Body weight

Each animal was weighed at least weekly during the acclimatization phase and then weekly for the first 13 weeks of the study, approximately every four weeks thereafter, and prior to necropsy.

3. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded for each animal. Food consumption was recorded weekly during the first 13 weeks of treatment, and once every four weeks thereafter.
The mean achieved dosage in mg/kg bw/day was calculated based on body weight and food consumption.

4. Hematology

Animals were diet-fasted overnight prior to anesthesia by isoflurane inhalation for blood collection by puncture of the retro-orbital venous plexus. Blood was collected into tubes containing EDTA and standard hematology parameters were determined. These analyses were conducted on all surviving animals of the interim sacrifice group, as well as the first ten surviving animals of the terminal sacrifice groups, in week 53, and on the first twenty surviving mice of the terminal sacrifice groups during week 80.

5. Test item analysis

At the end of month 3, at the end of the chronic phase in month 12, and at the end of the carcinogenicity phase in month 18, blood samples were collected from the retro-orbital venous plexus of five animals per sex from each treated group. Animals were not fasted overnight, but were anesthetized by isoflurane inhalation prior to blood sampling. Blood was collected into heparinized vials, and plasma was prepared by centrifugation prior to storage in the dark at approximately -20°C until analysis.

6. Sacrifice and pathology

On study days 377 to 379 for the 12-month interim kill, and on study days 553 to 568 for the carcinogenicity phase, all surviving animals in the relevant phases were sacrificed by exsanguination under deep anesthesia ensured by isoflurane inhalation. An approximately

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equal number of animals randomly distributed among all groups were sampled on each day. Animals were diet-fasted overnight prior to sacrifice.

All animals, including those either found dead or killed for human reasons, were necropsies. The necropsy included the examination of the external surfaces, all orifices, and all major organs, tissues, and body cavities. All significant macroscopic abnormalities were recorded, sampled, and examined microscopically.

Table 5.5/02-1: Organs and tissues which were sampled and / or weighed at necropsy

	Digestive system		**Cardiovasc. / Hemat.**		**Neurologic**
X	Tongue	X	Aorta thoracic*	XX	Brain (3 sections)*+
X	Submandibular (salivary) gland	XX	Heart*+		
		X	Bone marrow, sternum*	X	Sciatic nerve*
X	Esophagus*	X	Lymph node, mesenteric*	X	Spinal cord (cervical, thoracic, lumbar)*
X	Stomach*				
X	Duodenum*	X	Lymph node, submaxillary	X	Eyes (retina)*
X	Jejunum*			X	Optic nerves*
X	Ileum*	XX	Spleen*+		
X	Cecum*	X	Thymus		**Glandular**
X	Colon*			X	Pituitary gland*
X	Rectum*		**Urogenital**	XX	Adrenal gland*+
XX	Liver*+	XX	Kidney*+	X	Parathyroid gland*
X	Gall bladder*	X	Urinary bladder*	X	Thyroid gland*
X	Pancreas*	XX	Testis*+	X	Harderian gland
		XX	Epididymis*+		Lacrimal exorbital gland
	Respiratory	X	Prostate gland*		
X	Trachea*	X	Seminal vesicle*		
X	Lung*	XX	Ovary*+		**Other**
^	Pharynx*	XX	Uterus (with cervix)*+	X	Bone (sternum)
^	Larynx*	X	Mammary gland*	X	Skeletal muscle
^	Nasal cavities	X	Vagina	X	Skin*
				X	All gross lesions and masses
				X	Articular surface (femorotibial joint)

* required for carcinogenicity studies based on US EPA guideline 870.4200
\+ organ weights required for rodent studies
X tissues were collected for histological examination
XX organs were weighed fresh at scheduled sacrifice only, with paired organs weighed together, and collected for histological examination
^ organs were preserved for possible micropathologic examination only

Two femoral bone marrow smears were prepared from sacrificed animals (except on weekends and public holidays), one of which was stained with May-Gruenwald Giemsa, while the other smear was stored unstained. As there was no relevant change in either hematology or bone marrow histology, these smears were not examined.

All tissues specified above were embedded in paraffin wax. For the 12-month interim sacrifice animals, sections were prepared from the liver in all groups, and from thyroid gland in the control and high-dose groups. For the final sacrifice phase, sections were prepared from all organs and tissues for all animals.

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II. RESULTS AND DISCUSSION

A. MORTALITY

There was a slight increase in mortality in females of the carcinogenicity phase at 1250 ppm, although this increase was not statistically significant. There was no effect on mortality at 1250 ppm in males or at either 50 or 250 ppm in either males or females.

Table 5.5/02-2: Mortality rate in male and female mice in the carcinogenicity phase administered BCS-CN88460 via the diet for up to 18 months

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	50	250	1250	0	50	250	1250
Initial N, day 0	50	50	50	50	50	50	50	50
Killed for humane reasons	9	6	6	8	6	10	9	14
Found dead	0	0	0	1	0	2	1	1

B. CLINICAL OBSERVATIONS

There were no treatment-related clinical signs observed in males at any dose level at any time during the study, and no changes noted at weekly physical examination which were considered to be related to treatment.

C. BODY WEIGHT

There was no effect of treatment on either body weight or body weight gain at 50 or 250 ppm. At 1250 ppm, mean body weight was significantly reduced in both males, from day 36 onward, and in females, on most intervals from day 71 onward. Mean cumulative body weight gain was slightly decreased at 1250 ppm in both males and females throughout the study.

Table 5.5/02-3: Mean body weight and body weight gain in male and female rats administered BCS-CN88460 in the diet for up to two years

		BCS-CN88460, dietary concentration in ppm							
		Males				Females			
Measure	Day	0	50	250	1250	0	50	250	1250
Body weight, g	1	20.63	20.57	20.58	20.51	16.77	16.87	16.88	16.83
	8	21.98	21.98	21.70	21.63	18.00	18.03	17.73	17.41**
	92	27.90	27.47	27.74	27.05**	22.36	21.96	21.71**	21.23**
	176	30.61	30.63	30.61	29.20**	24.07	23.87	23.83	22.86**
	365	32.80	32.52	33.25	31.33†	26.98	27.02	26.86	25.43**
	540	33.11	33.19	32.95	31.72**	27.92	27.87	27.74	26.52*
Body weight gain, g	1-8	1.35	1.41	1.12*	1.12*	1.23	1.16	0.85**	0.57**
	1-92	7.28	6.90	7.14	6.54**	5.58	5.09**	4.83**	4.40**
	92-176	2.71	3.15*	2.83	2.15**	1.71	1.91	2.12*	1.64
	176-365	2.12	1.72	2.53	2.06	2.89	3.14	3.00	2.47
	365-540	0.30	0.54	-0.15	0.35	0.91	0.97	0.84	0.93
	1-540	12.51	12.49	12.35	11.14**	11.15	10.88	10.89	9.66**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; † $p \leq 0.001$.

D. FOOD CONSUMPTION

There was no effect on food consumption in either males or females at any dose in this study at any time point.

E. ACHIEVED INTAKE

Compound intake is shown in Table 5.5/02-3 for various periods of the study.

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Table 5.5/02-4: Mean intake of BCS-CN88460, in mg/kg bw/day in male and female rats treated via the diet

	BCS-CN88460, dietary concentration in ppm					
	Males			Females		
Weeks	**50**	**250**	**1250**	**50**	**250**	**1250**
1-13	6.8	34.1	172	8.6	42.3	212
1-52	6.1	30.2	154	8.1	39.6	197
1-80	5.9	29.0	147	7.8	38.1	190

F. HEMATOLOGY AND TEST ITEM ANALYSIS

1. Hematology

There were no changes in hematological parameters in either males or females which were related to dietary administration of BCS-CN88460.

2. Bioanalytical examinations

Plasma samples were analyzed for concentrations of BCS-CN88460 at 3 to 4 months of dietary administration of the compound, and for BCS-CN88460 as well as the metabolites BCS-CX99799 and BCS-CX99798 at 12 and 18 months of the study.

In the control group, concentrations for the parent compound and its metabolites were below the limit of quantification of 0.01 mg/L in both males and females.

The parent compound was detected below or only slight above the LOQ in both males and females throughout the study. Dose-related increases of the metabolites were generally observed in both sexes, although plasma concentrations were greater in females than in males.

Table 5.5/02-5: Mean plasma concentrations of BCS-CN88460, BCS-CX99799, and BCS-CX99798 in male and female rats administered BCS-CN88460 via the diet for up to two years

	BCS-CN88460, dietary concentration in ppm					
	Males			Females		
3 months	**50**	**250**	**1250**	**50**	**250**	**1250**
BCS-CN88460	< LOQ	< LOQ	< 0.0102	< LOQ	< LOQ	< 0.0184
12 months						
BCS-CN88460	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< 0.0198
BCS-CX99798	0.0294	0.125	0.486	0.0414	0.312	0.986
BCS-CX99799	0.0532	0.156	1.21	0.0692	0.590	6.50
18 months						
BCS-CN88460	< LOQ	< LOQ	< 0.0106	< LOQ	< LOQ	< 0.0254
BCS-CX99798	0.0334	0.134	0.584	0.0656	0.356	0.844
BCS-CX99799	0.0610	0.198	1.54	0.0638	0.552	4.40

G. SACRIFICE AND PATHOLOGY

1. Terminal body weight and organ weight

At the 12-month interim sacrifice, there was no effect on mean terminal body weight at any dose group. At 1250 ppm, absolute and relative liver weights were increased relative to control values.

At the 18-month terminal sacrifice, at 1250 ppm mean terminal body weight was significantly decreased in both males and females. At 1250 ppm, absolute and relative liver weights were increased in both males and females. Absolute and relative kidney weights were increased in males at 1250 ppm; this increase was considered to be related to treatment as it was associated with relevant microscopic findings. In contrast, the slight increase at

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250 ppm in absolute and relative kidney weights at 250 ppm was not associated with any microscopic changes and is thus considered to be incidental and not related to treatment. Absolute and relative adrenal weights were increased at 1250 ppm in both males and females, but in the absence of any relevant gross or histopathological changes, this increase in organ weight is considered to be incidental and not treatment-related.

Table 5.5/02-6: Mean terminal body weight and absolute and relative liver and kidney weight in male and female mice administered BCS-CN88460 via the diet for up to 18 months

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	50	250	1250	0	50	250	1250
12m, N ex.	9	9	9	10	10	9	10	9
Terminal body wt, g	29.1	29.1	30.6	27.6	23.5	23.8	22.9	22.4
Liver wt, g	1.169	1.204	1.237	1.338**	1.084	1.128	1.107	1.179
Liver wt / body wt, %	4.030	4.134	4.059	4.845 ***	4.611	4.741	4.842	5.270**
Liver wt / brain wt, %	251.98	259.30	261.84	284.97 **	226.25	239.88	232.37	252.86*
18m, N ex.	41	44	44	41	44	38	40	35
Terminal body wt, g	29.12	29.13	28.93	27.43 **	25.01	24.78	24.69	23.41**
Liver wt, g	1.200	1.266	1.200	1.322 ***	1.279	1.253	1.329	1.322
Liver wt / body wt, %	4.129	4.375	4.157	4.822 ***	5.089	5.065	5.393	5.651 ***
Liver wt / brain wt, %	258.82	271.72	258.74	284.72 ***	264.12	261.22	279.38	278.70
Adrenal wt, g	0.00419	0.00416	0.00446	0.00582 **	0.00787	0.00792	0.00782	0.00836
Adrenal wt / body wt, %	0.01416	0.01439	0.01556	0.01840 **	0.03162	0.03227	0.03188	0.03589 **
Adrenal wt / brain wt, %	0.88546	0.89015	0.96237	1.08092 **	1.63005	1.65627	1.64341	1.75977 *
Kidney wt, g	0.4915	0.4996	0.5175*	0.5336 **	0.4039	0.3937	0.3875	0.3804
Kidney wt / body wt, %	1.6857	1.7082	1.7916 **	1.9471 ***	1.6166	1.5981	1.5711	1.6269
Kidney wt / brain wt, %	105.9740	106.8918	111.5849 *	114.9555* **	83.8853	82.2196	81.3981	80.1813

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

2. Macroscopic findings

There were no macroscopic findings which were related to treatment in either males or females in the interim-sacrifice animals. In the terminal sacrifice animals, at 1250 ppm, the incidence of enlarged liver was increased compared to controls. However, this is considered to be not treatment-related as it was not associated with any increase in liver weight nor with any relevant microscopic findings.

3. Microscopic findings

In the interim-sacrifice animals, there were no neoplastic findings at the end of the 12-month treatment period.

Non-neoplastic findings were observed in the liver of both males and females in the interim sacrifice group. In males, the incidence of multinucleated hepatocytes was increased at

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1250 ppm, while in females the incidence of bile duct hyperplasia was increased at 1250 ppm.

Table 5.5/02-7: Microscopic findings in liver of male and female mice administered BCS-CN88460 via the diet for up to 12 months

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	50	250	1250	0	50	250	1250
N examined	10	10	10	10	10	10	10	10
Multinucleated hepatocytes								
Minimal	1	0	0	8	0	0	1	0
Total	1	0	0	8**	0	0	1	0
Bile duct hyperplasia: diffuse								
Minimal	1	0	0	0	0	0	1	3
Slight	0	0	0	0	0	0	1	0
Total	1	0	0	0	0	0	2	5**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

After 18 months dietary administration of BCS-CN88460, there were no treatment-related neoplastic findings in either males or females.

Non-neoplastic, treatment-related findings were noted at 1250 ppm in the liver of both males and females, and in the kidney of males. In males, there was a greater incidence and severity of multinucleated hepatocytes and single cell necrosis. In females, there was a greater incidence and severity of diffuse bile duct hyperplasia, a greater incidence of hepatocellular necrotic foci, and a lower incidence and severity of diffuse hepatocellular vacuolation when compared to controls.

Table 5.5/02-8: Microscopic findings in liver of male and female mice administered BCS-CN88460 via the diet for up to 18 months

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	50	250	1250	0	50	250	1250
N examined	50	50	50	50	50	50	50	50
Multinucleated hepatocytes								
Minimal	3	1	4	28	0	0	0	0
Slight	0	0	0	14	0	0	0	0
Moderate	0	0	0	1	0	0	0	0
Total	3	1	4	43***	0	0	0	0
Bile duct hyperplasia: diffuse								
Minimal	5	7	5	2	8	8	9	19
Slight	0	1	0	3	0	1	0	6
Total	5	8	5	5	8	9	9	25***
Single cell necrosis: focal								
Minimal	4	0	2	8	5	2	3	2
Slight	0	0	0	2	0	0	1	0
Total	4	0	2	10*	5	2	4	2
Hepatocellular necrotic focus(i)								
Minimal	11	9	7	14	12	9	13	22
Slight	0	0	0	1	0	0	0	2
Total	11	9	7	15	12	9	13	24*
Hepatocellular vacuolation: diffuse								
Minimal	29	29	31	27	13	15	15	18
Slight	8	8	5	8	25	21	21	12
Moderate	0	0	0	1	3	1	2	1
Total	37	37	36	36	41	37	38	31

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

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In the kidney of males, there was an increased incidence of hyaline casts and of tubule dilatation in the medulla, as well as a slightly increased severity of focal tubule basophilia when compared to controls.

Table 5.5/02-9: Microscopic findings in kidney of male and female mice administered BCS-CN88460 via the diet for up to 18 months

	BCS-CN88460, dietary concentration in ppm							
	Males				Females			
	0	50	250	1250	0	50	250	1250
N examined	50	50	50	50	50	50	50	50
Hyaline casts: focal								
Minimal	38	31	34	42	41	34	45	42
Slight	3	5	4	3	5	7	4	5
Moderate	0	0	0	1	1	0	0	0
Total	41	36	38	46**	47	41	49	47
Tubule dilation: medulla: focal								
Minimal	0	0	1	4	8	3	3	3
Total	0	0	1	4*	8	3	3	3
Tubule basophilia: focal								
Minimal	47	43	39	30	43	37	44	42
Slight	3	7	7	14	3	6	2	1
Moderate	0	0	1	2	0	0	0	0
Marked	0	0	0	1	0	0	0	0
Total	50	50	47	47	46	43	46	43

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Amyloid deposition was observed in almost all animals in various organs. However, at 1250 ppm the incidence and / or severity of amyloid deposition were observed in the liver of males and females, the submaxillary lymph node in females only, the kidney in females only, and the adrenal and thyroid gland of both males and females.

H. DEFICIENCIES

No deficiencies are identified in this study.

III. CONCLUSIONS

Dietary administration of BCS-CN88460 over an 18-month period to the C57BL/6J mouse, at dose levels up to 1250 ppm (equivalent to 147 mg/kg bw/day in males and 190 mg/kg bw/day in females) did not induce carcinogenic effects. Based on the observation of increased liver weight and histopathological findings, the NOAEL was considered to be 250 ppm (equivalent to 29.0 mg/kg bw/day in males and 38.1 mg/kg bw/day in females).

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CA 5.6 Reproductive toxicity

Table 5.6-1: Summary of reproductive and developmental toxicity studies with Isoflucypram

Study	NOAEL	LOAEL	Effects
2-generation reproduction study Dietary study 0, 150, 450, 1200 ppm (concentrations halved during lactation) [redacted], R.; 2018; M-612750-02-1	1200 ppm approximately 90 mg/kg bw/day (see Table 5.6.1/01-4 for details)	> 1200 ppm	Increased liver weight without accompanying microscopic findings
Rat developmental toxicity study Oral gavage dosing 0, 25, 125, 625 mg/kg bw/day [redacted]; 2017; M-602126-01-1	Maternal and fetal: 125 mg/kg bw/day	Maternal and fetal: 625 mg/kg bw/day	Maternal: decreased body weight / body weight gain, increased liver weight, altered clinical chemistry parameters Fetal: decreased fetal body weight, slight delays in ossification
Rabbit developmental toxicity study Oral gavage [redacted]; 2017; M-588469-01-1	Maternal: 70 mg/kg bw/day Fetal: 500 mg/kg bw/day	Maternal: 500 mg/kg bw/day Fetal: > 500 mg/kg bw/day	Maternal: Body weight loss, reduced food consumption, increased mean liver weight Fetal: no effects

In the two-generation reproduction study conducted via the diet, concentrations of BCS-CN88460 were 0, 150, 450, and 1200 ppm during pre-mating phases of the study, and for females were 0, 75, 225, and 600 ppm during the lactation phase so that the achieved dose would remain constant during all phases of the study. There was no effect of treatment on clinical signs or body weight, although food consumption was slightly decreased during the pre-mating phase of the F1 adult animals. Estrous cyclicity, pre-coital interval, mating performance and fertility, gestation length, number of implantations, litter size, sex ratio of the offspring, survival of offspring through weaning, sperm assessment (motility, morphology, or concentration), ovarian follicle count, or anogenital distance (in the F2 offspring) were not affected by dietary administration of BCS-CN88460. The age of and body weight at sexual maturation were slightly increased in female F1 pups, although there was no effect on either fertility or fecundity of these animals. Absolute and relative liver, thyroid, and / or kidney weights were increased in at least one generation in adults and / or offspring. Microscopic examination of liver, thyroid, and kidney in adult animals did not show any treatment-related effects. The NOAEL of this study, for reproductive performance of the F0 and F1 adults, and for the survival, growth, and development of the F1 and F2 offspring, is 1200 ppm for adult males and 1200 / 600 ppm for adult females and for offspring.

In the rat developmental toxicity study, BCS-CN88460 was administered by oral gavage at 0, 25, 125, and 625 mg/kg bw/day to pregnant rats from gestation days 6 to 20 inclusive. There were no clinical signs or mortalities. Body weight, body weight gain, and corrected body weight gain were slightly decreased at 625 mg/kg bw/day, as was food consumption. Absolute liver weight, enlarged liver, and the incidence of hepatocellular hypertrophy were increased only at the top dose of 625 mg/kg bw/day. Thyroid weight was not affected, but one animal at 625 mg/kg bw/day showed thyroid follicular cell hypertrophy. There was no effect of treatment on pregnancy rate or on pre- or post-implantation or fetal data, other than a slight decrease in fetal body weight at 625 mg/kg bw/day. The incidence of

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fetal malformations was not increased by maternal administration of BCS-CN88460. At 625 mg/kg bw/day, at visceral examination, there was a slight increase in the incidence of distended bladder and of renal pelvis dilation, as well as a slight decrease or delay in ossification of a number of structures at skeletal examination. The maternal and fetal NOAEL was 125 mg/kg bw/day.

In the rabbit developmental toxicity study, BCS-CN88460 was administered by oral gavage at 0, 10, 70, and 500 mg/kg bw/day to pregnant rabbits from gestation days 6 to 28 inclusive. One abortion observed on gestation day 26 may have been indirectly related to treatment due to the poor health status of the animal. There were no other treatment-related mortalities nor were any clinical signs observed. Overall body weight gain was reduced at 500 mg/kg bw/day, with some decrease in body weight gain observed at 80 mg/kg bw/day between gestation days 6 and 10. Liver weight was increased at 500 mg/kg bw/day. Pregnancy rate, pre- or post-implantation data and fetal data were not affected in any group. There were no treatment-related malformations, and no findings at external, visceral, or skeletal examination. The maternal NOAEL for this study was 70 mg/kg bw/day, while in the absence of any fetal findings the fetal NOAEL was 500 mg/kg bw/day, the highest dose tested.

CA 5.6.1 Generational studies

Report:	KCA 5.6.1/01; [redacted], R.; 2018; M-612750-02-1
Title:	BCS-CN88460 technical: Two generation reproductive performance study by dietary administration to han wistar rats
Report No.:	M-612750-02-1
Document No.:	M-612750-02-1
Guideline(s):	OPPTS Guideline No. 870.3800 JMAFF, 12 Nohsan No. 8147 OECD Guideline No. 416
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

The objective of this study was to assess the influence of BCS-CN88460 technical (a fungicide) on reproductive performance when continuously administered via the diet through two successive generations of Han Wistar rats.

For the F0 generation, three groups of 28 male and 28 female rats received BCS CN88460 technical orally, via the diet, at concentrations of 150, 450 or 1200 ppm for ten weeks before pairing, throughout pairing and gestation. During the lactation phase, the dietary concentrations were lowered by 50% to 75, 225 and 600 ppm. A similarly constituted Control group received untreated basal diet for the same duration. The F1 generation comprised of 24 male and 24 female progeny from each group, and they continued to receive the relevant diet, as per the F0 generation, throughout the study until termination. During the study, data were recorded on clinical condition, body weight, food consumption, blood chemistry, estrous cycles, mating performance and fertility, gestation length and parturition observations and reproductive performance. Sperm analysis, organ weight, macroscopic and microscopic pathology investigations were undertaken on each adult generation. The clinical condition of offspring, litter size and survival, sex ratio, sexual maturation (selected F1 generation only), body weight gain assessed, ano-genital distance were recorded and organ weight, macroscopic pathology investigations were undertaken on each generation.

Results

BCS-CN88460 technical was administered continuously to Han Wistar rats via the diet at dose levels of 150, 450 and 1200 ppm (adjusted to 75, 225 and 600 ppm for females during lactation) and was generally well tolerated, with no adverse effects on the clinical condition or survival of both adults and offspring. There was no adverse effect on adult bodyweights or food consumption.

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Biochemical examination of blood plasma revealed in both the F0 and F1 adults: higher calcium and total protein, a low albumin globulin ratio among males receiving 1200 ppm and higher cholesterol among females receiving 1200/600 ppm.
Oestrus cycles, pre-coital interval, mating performance and fertility were unaffected by treatment during each generation. There were no adverse effects on the age of sexual maturation for the F1 males or females.

There were no effects of treatment on litter size, offspring survival, sex ratio or offspring bodyweight in both generations or on ano-genital distance of F2 offspring.

Organ weight analysis revealed higher liver weights in both F0 and F1 adults in males and females receiving 1200/600 ppm and in females receiving 450/225 ppm. Among F1 and F2 offspring higher liver weights were recorded among males and females receiving 1200/600 or 450/225 ppm and in females receiving 150/75 ppm.
There were no macroscopic or histopathological findings in the F0 and F1 adults which were considered to be related to treatment with BCS-CN88460 technical.
There were no macroscopic findings in the F1 and F2 offspring which were considered to be related to treatment with BCS-CN88460 technical.

Based on the results of this study, it is concluded that treatment of two successive generations of Han Wistar rats at dietary concentrations of up to 1200 ppm (males) or 1200/600 ppm (females and offspring) BCS-CN88460 technical was well tolerated by both the F0/F1 parent animals and the F1/F2 offspring.

Accordingly, the NOAEL for reproductive performance of the F0/F1 adults, and the NOAEL for the survival, growth, and development of the F1/F2 offspring, is concluded to be 1200 ppm for adult males and 1200/600 ppm for adult females and offspring.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% (w/w)
CAS #	1255734-28-1
Stability of test compound:	Until 28 April 2016

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	rat
Strain:	RccHanTM Wistar rat
Age:	F0 animals were 40-47 days of age at the start of treatment
Weight at dosing:	131-187g for males; 105-145g for females
Source:	[redacted]
Acclimation period:	19 days before the start of treatment
Diet:	[redacted] certified powdered diet
Water:	Potable water from the public supply via polycarbonate bottles with sipper tubes

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Housing:	Cages with polycarbonate body and stainless steel mesh lid; during pairing, the cages used were grid-bottomed cages, while in all other phases solid-bottomed cages were used. During the pre-mating phase and for males through termination, up to four animals of one sex were housed together. During pairing, one male and one female were housed together. After pairing, females were housed individually until weaning; then up to four females were housed together until sacrifice. During offspring maturation until selection, all pups in one litter were housed together.
Environmental conditions:	
Temperature:	19-23°C
Humidity:	40-70%
Air changes:	Not described, but provided air was filtered fresh air which was not recirculated.
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN

1. **In life dates:** 4 June 2015-9 March 2016

2. **Animal assignment and treatment**
During the acclimatization period of the animals which formed the F0 group, all litters were assessed and animals were weighed individually. The method of allocation of animals to the F0 generation ensured that no more than one offspring of each sex from each litter was present in each group. Animals showing signs of ill health were excluded, and animals at the extreme of the weight range or litters showing large variations in individual weights were not selected if alternatives were available.

For selection of offspring to form the F1 generation, after exclusion of grossly atypical animals, the offspring with the lowest within-litter identification per sex from each selected litter were selected to form the F1 generation. Where possible, one male and one female were selected from each litter. Selected animals were tattooed on day 20 and separated from littermates on day 21 of age. Formal commencement of the F1 generation was on nominal day 28 of age, which where possible was 28 ± 2 days of age for selected F1 animals.

3. **Dose selection rationale**
In the preliminary one-generation reproduction study (Envigo study number: DNM0082), males and females were administered diets containing BCS-CN88460 at concentrations of 0, 100, 300, and 1000 ppm for four weeks before pairing as well as during pairing and gestation. During the lactation phase, the dietary concentrations for females were reduced by 50% to maintain the systemic dose in mg/kg bw/day at a relatively constant value. The dietary concentrations used in this study were the same ones used in the 90-day rat study, as it was assumed that those dietary concentrations would produce a range of effects from none at 100 ppm to significant toxicity at 1000 ppm. However, there were no effects seen with regard to clinical signs, body weight or body weight gain, estrus cyclicity, pre-mating interval, fertility, fecundity in the parents, and no effects observed on body weight gain or sexual maturation of the offspring. The only treatment-related effects were:

- At 1000 ppm, an increase in adjusted liver weight in F0 males and F1 males and females, and
- At 300 ppm, an increase in adjusted liver weight in F1 males.

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Based on these results, the dietary concentrations selected for the two-generation study were 0, 150, 450, and 1200 ppm. The concentrations of 150 and 450 ppm were chosen as they corresponded to concentrations used in the rat chronic study. The dietary concentration of 150 ppm was expected to be a NOAEL, while some toxicity was expected at 450 ppm, and the highest concentration used, of 1200 ppm, was expected to produce significant systemic toxicity in both males and females.

During the lactation phase the dietary concentration for females was lowered by 50% to 75, 225 and 600 ppm so that the achieved dose for the F0 females in the high dose group does not increase markedly above the limit dose of 1000 mg/kg/day as the maternal food consumption increases.

4. Diet preparation and analysis

The required amount of BCS-CN88460 was added to an approximately equal amount of fine sieved diet by gentle stirring. An amount of sieved diet approximately equal to the mixture was added and the mixture was stirred until it appeared visibly homogeneous. This doubling-up procedure was repeated until approximately half of the premix weight was achieved. This mix was then ground using a mechanical grinder and made up to the required weight using the remaining coarse diet from the sieving process. At this stage, the mixture was mixed in a Turbula mixer for 200 cycles at 16 rpm to ensure all the test material was dispersed in the diet. Aliquots of the appropriate premix were diluted with further diet to produce the required dietary concentrations for feeding to the animals. Each batch of treated diet was mixed for a further 200 revolutions at 16 rpm in the Turbula mixer. Diet was prepared weekly and stored at ambient temperature.

Before commencement of treatment, the suitability of the proposed mixing procedures was determined and specimen formulations were analyzed to assess the stability and homogeneity of the test substance in the diet. Specimen formulations prepared at nominal concentrations of 50 ppm and 1000 ppm were prepared and analyzed as part of the preliminary study. Stability and homogeneity were confirmed at these concentrations for up to 15 days when stored at ambient (nominally 21°C) temperature and for 22 days when frozen (nominally -20°C). Further homogeneity and stability trials were conducted at the high dose level (1200 ppm) as part of this study, and both stability and homogeneity were confirmed at 1200 ppm for up to 22 days when stored at ambient temperature (nominally 21°C) and for 22 days when frozen (nominally -20°C).

Samples of each formulation prepared for administration in week 1 of the F0 generation, the F0 and F1 pairing phases, and the final week of lactation for the F0-F1 and for the F1-F2 generations were analyzed for achieved concentration of the test item.

5. Statistics

Statistical analyses were performed on the majority of data presented and results of these tests, whether significant or non-significant, are presented on the relevant tables. For some parameters, including estrous cycles (F1), pre-coital interval, mating performance, fertility, stage of estrous at termination, sperm analysis, motility, morphology and count, the similarity of the data were such that analyses were not considered to be necessary.

All statistical analyses were carried out separately for males and females. Data relating to food consumption were analyzed on a cage basis. For all other adult parameters, the analyses were carried out using the individual animal as the basic experimental unit. For litter/fetal findings the litter was taken as the treated unit and the basis for statistical analysis and biological significance was assessed with relevance to the severity of the anomaly and the incidence of the finding within the background control population.

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The following data types were analyzed at each timepoint separately:

- Body weight, using absolute weights and gains over appropriate study periods
- Food consumption, over appropriate study periods
- Blood chemistry
- Estrous cycles (F0)
- Gestation length
- Ano-genital distance
- Litter size and survival indices
- Sexual maturation, age and body weight at completion
- Organ weights, either absolute and relative to terminal body weight

The following sequence of statistical tests was used for body weight, food consumption, blood chemistry, ano-genital distance, litter size, survival indices, and sexual maturation and organ weight data:

A parametric analysis was performed if Bartlett's test for variance homogeneity was not significant at the 1% level. For pre-treatment data, analysis of variance was used to test for any group differences. Where this was significant ($p<0.05$) inter group comparisons using t-tests, with the error mean square from the one-way analysis of variance, were made. For all other comparisons the F1 approximate test was applied. This test is designed to detect significant departure from monotonicity of means when the main test for the comparison of the means is a parametric monotonic trend test such as Williams' test. The test statistic compares the mean square, NMS, for the deviations of the observed means from the maximum likelihood means, calculated under a constraint of monotonicity with the usual error mean square, EMS. The null hypothesis is that the true means are monotonically ordered. The test statistic is F1 = NMS/EMS which can be compared with standard tables of the F-distribution with 1 and error degrees of freedom. If the F1 approximate test for monotonicity of dose-response was not significant at the 1% level, Williams' test for a monotonic trend was applied. If the F1 approximate test was significant, suggesting that the dose response was not monotone, Dunnett's test was performed instead. Where there were only two groups, comparisons were made using t-tests.

A non-parametric analysis was performed if Bartlett's test was still significant at the 1% level following both logarithmic and square-root transformations. For pre treatment data, Kruskal-Wallis test was used to test for any group differences. Where this was significant ($p<0.05$) inter-group comparisons using Wilcoxon rank sum tests were made. For all other comparisons the H1 approximate test, the non-parametric equivalent of the F1 test described above, was applied. This test is designed to be used when the main test for comparison of the means is a non-parametric monotonic trend test, such as Shirley's test. The test statistic compares the non-monotonicity sums of squares, NRSS, for the deviations of the observed mean ranks from the maximum likelihood mean ranks with the non-parametric equivalent of the error sums of squares, ERSS = N(N+1)/12. The test statistic is H1 = NRSS/ERSS which can be compared to standard tables of the χ2-distribution with 1 degree of freedom. If the H1 approximate test for monotonicity of dose-response was not significant at the 1% level, Shirley's test for a monotonic trend was applied. If the H1 approximate test was significant, suggesting that the dose-response was not monotone, Steel's test (Steel 1959) was performed instead. Where there were only two groups, comparisons were made using Wilcoxon rank sum tests.

For blood chemistry, litter size, survival indices, ano-genital distance and sexual maturation data, if 75% of the data (across all groups) were the same value, Fisher's Exact tests were performed. Treatment groups were compared using pairwise comparisons of each dose group against the control both for i) values <c versus values ≥c, and for ii) values ≤c versus values >c, as applicable.

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Pre-/post-implantation loss and sex ratio were analyzed by generalized mixed linear model with binomial errors, a logit link function and litter as a random effect. Each treated group was compared to control using a Wald chi-square test. For pre-implantation loss, the numerator was Number of corpora lutea - Number of implantations, the denominator was Number of corpora lutea. For post-implantation loss, the numerator was Number of implantations - Number of live fetuses, the denominator was Number of implantations. For sex ratio, the numerator was Number of males, the denominator was Number of live fetuses. For resorptions, each treated group was compared to control by exact Wilcoxon rank sum test.

For gestation length an exact two-tailed Linear-by-linear test, with equally spaced scores, was applied to all groups. If the test was statistically significant ($p<0.05$), the highest dose group was excluded and the test re-applied. This 'step-down' process was repeated until the test was no longer statistically significant ($p\geq0.05$). If the exact version of the Linear-by-linear test could not be calculated (due to the size of the table containing the data), then the asymptotic version was used instead.

For estrous cycles an exact one-tailed (upper-tail) Linear-by-linear test was applied to all groups, using scores appropriate to the severity of the observation assuming 4 day cycles to be normal. The categories were scored as follows: a regular 4 or 5 day cycle was scored as 4.5 and irregular and acyclic cycles were scored as 6. If the test was statistically significant ($p<0.05$), the highest dose group was excluded and the test re-applied. This 'step-down' process was repeated until the test was no longer statistically significant ($p\geq0.05$). If the exact version of the Linear-by-linear test could not be calculated (due to the size of the table containing the data), then the asymptotic version was used instead.

C. METHODS

1. Clinical signs and mortality

A viability check was performed near the start and end of each working day, and animals were killed for reasons of animal welfare where necessary or if they exhibited pregnancy loss.

Animals were also inspected at least twice daily for evidence of ill health or reaction to treatment. Cages and cage trays were inspected daily for evidence of animal ill health. Any deviation from normal was recorded at the time in respect of nature and severity, date and time of onset, and duration and progress of the observed condition.

A detailed physical examination was performed once each week on all F0 and selected F1 animals, on days 0, 5, 12, 18, and 20 after mating, and on days 1, 7, 14, 21, and 28 during lactation.

2. Body weight

The body weight of animals was recorded as follows:

- F0 males: on the day when treatment commenced, each week, and at necropsy;
- F0 females: on the day that treatment commenced, each week until mating was detected, on days 0, 7, 14, and 20 after mating, and on days 1, 4, 7, 14, 21, 25, and 28 postpartum;
- F1 animals: at the same frequency as F0 animals following selection at nominally four weeks of age.

3. Food consumption and compound intake

The weight of food supplied to each cage, that remaining, and an estimate of any spillage was recorded as follows:

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- F0 adults: weekly until paired for mating
- F0 females: days 0-6, 7-13, and 14-19 after mating, and days 1-3, 4-6, 7-13, and 14-20 of lactation;
- F1 animals: at the same frequency as F0 animals following selection at nominally four weeks of age.

4. Parental animal observations and treatment

Estrous cyclicity was determined for adult females of the F0 and F1 generation via both dry and wet smears. Dry smears were taken daily for 22 days before pairing, using cotton swabs moistened with saline. The smears were examined to establish the duration and regularity of the estrous cycle. After pairing with the males, wet smears were taken daily using pipette lavage until evidence of mating was observed. Finally, for four days before scheduled termination (nominally days 25 to 28 postpartum, daily vaginal smears were taken to determine the stage of the estrous cycle at termination. For females whose litters had previously died, smears were taken on a theoretical days 25-28. Females that failed to either litter or mate were retained and smeared for four days starting on the day on which the first batch of "true" day 25 females started smearing, then killed with that first batch of females.

The F0 pairing commenced after 10 weeks of treatment, and the F1 pairing commenced at least 10 weeks after selection of the F1 offspring. Pairing was organized to avoid pairing of siblings, and lasted for up to two weeks. Daily checks were carried out for ejected copulation plugs in the cage trays, and day 0 of gestation was set as the day on which positive evidence of mating was detected. Males and females were separated on the day on which mating evidence was detected. The pre-coital interval was calculated for each female as the time between the first pairing and detection of evidence of mating.

The duration of gestation was set as the time that elapsed between mating and commencement of parturition. From day 20 after mating, females were checked three times daily for evidence of parturition. The progress and completion of parturition was monitored, numbers of live and dead offspring were recorded, and any difficulties observed were noted.

5. Litter observations

All litters were examined at approximately 24 hours after birth (day 1 of age) and then daily thereafter. Offspring were numbered individually on day 1within each litter using a toe tattoo. The anogenital distance was measured in F2 offspring on day 1.

On day 4, litters containing more than eight offspring were reduced to eight by random culling, leaving four males and four females in each litter wherever possible. The sex ratio of each litter was recorded on study days 1, 4 (before and after culling), and 21. Individual offspring body weights were recorded on study days 1, 4 (before and after culling), 7, 14, 21, and 25 of age. On day 21 of age, the dam was removed from the litter age and the offspring were weaned. Offspring to form the F1 generation were selected on study day 20, and the selected animals were separated from their litter mates on study day 21.

Sexual maturation was assessed by daily examination in males from day 38 of age and in females from day 25 of age until either preputial separation or vaginal opening occurred. Body weight was recorded on the day the landmark was reached.

6. Parental post-mortem observations

Adult F0 and F1 males were sacrificed after approximately 17 weeks of age, when the majority of litters had weaned, while the adult females were sacrificed on day 28 postpartum. Females failing to mate or produce viable litters, and those with total litter loss, were retained until scheduled sacrifice with the first females reaching day 28 postpartum. The animals were killed by inhaled carbon dioxide, and subjected to a complete

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macroscopic examination. Selected organs were weighed and / or prepared for light microscopy. In addition, samples for sperm analysis were taken from males and from females uteri were examined to determine the number of implantation sites.

Table 5.6.1/01-1: Organs and tissues sampled at necropsy of adults in the 2-generation reproduction study

Tissue or region	Necropsy		Histology	Light microscopy
	Weigh	Fix		
Abnormalities		*	*	*
Adrenals	*	*	*	*
Brain (cerebellum, cerebrum, midbrain)	*	*	*	#
Epididymides (caput, cornus, cauda)	L&R	R	R	R
Kidneys	*	*	*	#
Liver (section from two lobes)	*	*	*	#
Mammary area (caudal)		a)	#	#
Ovaries	L&R	b) L&R	b) L&R	L&R
Pituitary	*	*	*	*
Prostate	*	*	*	*
Seminal vesicles (w/ coagulating gland)	*	*	*	*
Spleen	*	*	#	#
Testes	L&R	R	R	R
Thymus	*	*	#	#
Thyroid with parathyroid	c) *	*	*	*
Uterus with cervix and oviducts	*	*	*	*
Vagina		*	d) *	*

L&R bilateral organs weighed separately
* organs weighed, samples fixed or sections examined microscopically
# examined if effects suspected during the study

a) Females with litter death
b) Fixed identified as L&R. Five sections cut at approximately 100-micron intervals from the inner third of each ovary
c) Weighed after partial fixation
d) Section approximately 5 mm from vulva

Immediately after scheduled sacrifice of each F0 and selected F1 male, the left vas deferens, epididymis, and testis were removed and the epididymis and testis were weighed. A sample of sperm was expressed from the vas deferens into prewarmed medium M99, which contained 0.5% (w/v) bovine serum albumin (BSA Fraction V). A sample for assessment was taken into a 100 µm depth cannula by capillary action and at least 200 sperm per animal were analyzed using computer assisted sperm analysis (CASA).

For animals in the control and 1200 ppm groups, a 200 µL aliquot of the sperm/medium mixture described above was diluted with 800 µL of 10% neutral buffered formalin. After staining with nigrosine and eosin, an air-dried smear was prepared, and slides were examined by light microscopy for the assessment of morphology of at least 200 sperm from each male.

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For animals in the control and 1200 ppm groups, the left cauda epididymis of each male was weighed and the tunica was removed. The portion obtained was weighed, then homogenized for at least thirty seconds in 10 mL of a mixture of 0.9% saline and 0.01 merthiolate (SM). An aliquot of this mixture was added to a pre-prepared IDENT stain tube before being assessed for sperm count using CASA. After removal of the tunica, the left testis of each male was homogenized for at least thirty seconds in 25 ml of SM. An aliquot of this mixture was added to a pre-prepared IDENT stain tube before being assessed for homogenization-resistant spermatid count using CASA.

7. Offspring post-mortem observations

Offspring younger than 14 days of age were killed by intraperitoneal injection of sodium pentobarbitone, while above 14 days of age animals were killed by inhalation of carbon dioxide. Culled offspring sacrificed at the age of 4 days were examined externally; those which were externally normal were discarded without further examination, while externally abnormal offspring were examined internally and any abnormal tissues were retained. On day 21, one male and one female were selected at random from each litter, body weight was recorded, and specific organs were weighed and fixed. Abnormalities were fixed and retained for all offspring.

Table 5.6.1/01-2: Organs and tissues sampled at necropsy of offspring in the 2-generation reproduction study

Tissue or region	Necropsy		Histology	Light microscopy
	Weigh	Fix		
Abnormalities		*	$	#
Brain	*	*	#	#
Epididymides		*	#	#
Liver	*	*	#	#
Ovaries		*	#	#
Prostate		*	#	#
Seminal vesicles (w/ coagulating gland)		*	#	#
Spleen	*	*	#	#
Testes		*	#	#
Thyroid with parathyroid	a)	*	#	#
Thymus	*	*	#	#
Uterus with cervix and oviducts	*	*	#	#
Vagina		*	#	#

* organs weighed, samples fixed or sections examined microscopically
a) weighed after fixation
$ consideration was given to offspring abnormalities; only those deemed appropriate were examined.
# examined if effects were suspected during the study.

8. Clinical chemistry and test compound analysis

In week 9 of the pre-pairing period in the F0 and F1 generation, the ten lowest-number animals in each group were fasted overnight for clinical chemistry determinations. Animals were anesthetized by isoflurane inhalation, and blood samples were withdrawn from the sublingual vein into tubes containing lithium heparinate. After separation of the plasma, clinical chemistry parameters were measured.

In the F0 and F1 generation animals, at the end of treatment for the adult males, on day 18 of lactation for the F0 and F1 females, and on day 21 of age for unselected F1 and F2 offspring, three males and three females per group were used for blood sampling for bioanalysis. The animals were anesthetized by lithium inhalation and blood samples were

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taken from the orbital sinus into vials containing lithium heparinate. The samples were centrifuged at 2000g for 10 minutes at 4°C, and the plasma was held at -20°C until shipping for analysis.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no treatment-related effects on mortality in the adult F0 or the F1 animals.

B. OBSERVATIONS

1. Clinical signs

No clinical signs related to treatment were observed in either males or females of the F0 or F1 generations.

2. Clinical chemistry

In the F0 generation, bile acids were statistically significantly decreased in males at 450 and 1200 ppm, with a statistically non-significant decrease observed in females. Total cholesterol was statistically significantly increased in females at 1200 ppm, and statistically non-significantly increased in males at 1200 ppm. In the F1 generation, cholesterol was increased in males from 450 ppm and in females at 1200 ppm. These findings are characteristic of a compound acting through the CAR-PXR mode of action.

Table 5.6.1/01-3: Selected clinical chemistry findings for male and female rats administered BCS-CN88460 via the diet

		BCS-CN88460, dietary concentration in ppm							
		Males				Females			
Gen	Para-meter	0	150	450	1200	0	150	450	1200
F0	Bilirubin µmol/L	1	1	1	1	1	1	1*	1*
	Bile acids µmol/L	41.4	34.9	26.2**	25.6**	39.4	46.4	27.8	24.6
	Cholesterol mmol/L	2.03	2.07	2.09	2.27	1.75	1.93	1.97	2.56**
F1	Bilirubin µmol/L	1	1	1	1	1	1	1	1
	Bile acids µmol/L	38.2	38.3	43.4	21.0	30.3	27.0	18.3	17.5
	Cholesterol mmol/L	1.72	1.61	2.03**	1.97**	1.60	1.66	1.95	2.07*

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

C. BODY WEIGHT

In the F0 generation, there was no effect on body weight of males at any dose. In females, during the pre-mating period there was no effect on body weight or body weight gain. During the gestation phase, female body weight did not differ from controls. Mean overall body weight gain during lactation did not change relative to controls at any dose level, although there were slight but statistically significant variations during days 1-4 and days 7-14.

In the F1 generation, there was no effect on body weight during any phase of treatment in either males or females.

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There was no effect on the body weight or body weight gain of offspring in either the F1 or the F2 generations.

D. FOOD CONSUMPTION

Food consumption in the F0 males and females was not changed as a result of dietary BCS-CN88460 administration.

In the F1 generation, during weeks 5-10 of the pre-mating phase, at 1200 ppm there was a marginal decrease in food consumption compared to controls. A marginal decrease in food consumption was also seen in females at 150 and 450 ppm during week 10 only. There was no effect on food consumption in the males at any dose level.

E. COMPOUND INTAKE

The compound intake in each phase of the study is shown in Table 5.6.1/01-4.

Table 5.6.1/01-4: Achieved dose, in mg/kg bw/day at various phases of the 2-generation reproduction study conducted with BCS-CN88460

		BCS-CN88460, dietary concentration in ppm					
		Males			Females[1]		
Phase	**Generation**	**150**	**450**	**1200**	**150**	**450**	**1200**
Pre-mating	F0	11.27	34.1	94.4	13.03	40.8	140.4
	F1	12.91	41.6	108.6	14.62	44.5	112.5
Gestation	F0				11.69	35.9	94.7
	F1				12.94	38.4	102.5
Lactation	F0				11.49	35.8	94.5
	F1				11.28	34.1	92.9

1 Dietary concentrations were reduced during gestation and lactation to 75, 225, and 600 ppm in order to maintain a constant mg/kg bw/day dose.

F. REPRODUCTIVE FUNCTION

1. Estrous cyclicity

In the F0 and F1 generations, there were no clear effects of BCS-CN88460 administration on estrous cycles at any dose. The assessment of vaginal cytology on day 28 postpartum in both generations did not reveal any differences across the groups that could be associated with treatment.

2. Pre-coital interval

There was no effect of treatment in either the F0 or the F1 generation on the pre-coital interval.

3. Mating performance and fertility

There was no effect on either mating performance or fertility at any dose in the F0 animals.

4. Gestation length and gestation index

Both gestation length and gestation index were unaffected by dietary administration of BCS-CN88460 in the F0 generation.

In the F1 generation, there was a slight decrease in gestation length at 1200/600 ppm, although gestation index was unaffected.

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Table 5.6.1/01-5: Distribution of gestation duration, and gestation index in F0 and F1 rats administered BCS-CN88460 via the diet

		BCS-CN88460, dietary concentration in ppm			
Gen	**Parameter**	**0**	**150 / 75**	**450 / 225**	**1200 / 600**
F0	%, gestation = 22 days	25	32	25	14
	%, gestation = 22.5 days	25	18	38	39
	%, gestation = 23 days	50	50	38	46
	Gestation index	100	100	96	100
F1	%, gestation = 22 days	17	9	33	29
	%, gestation = 22.5 days	48	65	42	58
	%, gestation = 23 days	35	26	25	13
	Gestation index	100	100	100	100

5. Litter size, sex ratio, and offspring survival

The number of implantations, the litter size, the sex ratio of the offspring, and the survival of offspring through weaning were not affected by treatment in the F0 and F1 generations.

6. Sperm assessment

In the F0 and F1 generations, there was no effect of BCS-CN88460 administration on sperm motility, morphology, or concentration.

7. Ovarian follicle count

The ovarian follicle count in F1 females at 1200 ppm was similar to that observed in controls.

8. Anogenital distance

There was no effect on the anogenital distance in the F2 offspring.

9. Sexual maturation

There was no treatment-related effect on preputial separation in the F1 males. In females, age and body weight at vaginal opening were both statistically significantly increased at 1200/600 ppm. This is however considered not to represent an adverse effect of treatment, as subsequent estrous cycles, mating performance, fertility, and reproductive performance (litter size, offspring survival, and development), organ weights, macroscopic and microscopic appearance of female reproductive organs, and ovarian primordial follicle counts of these females were unaffected by treatment.

Table 5.6.1/01-6: Group mean age and body weight at sexual maturation in F1 pups administered BCS-CN88460 via the diet

		BCS-CN88460, dietary concentration in ppm			
Sex	**Parameter**	**0**	**150 / 75**	**450 / 225**	**1200 / 600**
Males	Age, days	46	46	47	48
	Body weight, g	196	192	196	202
Females	Age, days	33	33	33	38**
	Body weight, g	105	103	104	124**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

G. NECROPSY

1. Organ weights

In the F0 generation, absolute and relative liver and thyroid weights, and relative kidney weights, were increased in males. In females and offspring, absolute and relative liver weights were increased, while at the top dose both absolute and relative thymus weights were decreased.

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In the F1 generation, absolute and relative liver weights were increased in both males and females. Absolute thymus weights were decreased in males at the top dose.

In the F2 offspring, absolute and relative liver weights were increased in both male and female pups on post-natal day 21.

The increased liver and thyroid weights are consistent with the CAR-PXR mode of action of BCS-CN88460.

Table 5.6.1/01-7: Mean terminal body weight and absolute and relative weights of selected organs in male rats in the 2-generation reproduction study conducted with BCS-CN88460

			BCS-CN88460, dietary conc. in ppm			
Gen	**Phase**	**Parameter**	**0**	**150**	**450**	**1200**
F0	Week 17	Terminal body wt, g	438	444	439	430
		Liver wt, g	14.45	14.45	15.01	15.94**
		Liver wt, % body wt	3.30	3.25	3.42	3.70**
		Thyroid wt, g	0.017	0.018	0.020*	0.019*
		Thyroid wt, % body wt	0.0038	0.0041	0.0045*	0.0045**
		Thymus wt, g	0.285	0.276	0.290	0.286
		Thymus wt, % body wt	0.0650	0.0623	0.0663	0.0666
F1	PND 21	Terminal body wt, g	48.3	48.4	49.2	49.3
		Liver wt, g	2.174	2.272	2.408*	2.551**
		Liver wt, % body wt	4.513	4.671	4.874**	5.157**
		Thyroid wt, g	0.0056	0.0056	0.0058	0.0058
		Thyroid wt, % body wt	0.0116	0.0117	0.0117	0.0118
		Thymus wt, g	0.202	0.204	0.208	0.206
		Thymus wt, % body wt	0.419	0.423	0.424	0.418
	Week 17	Terminal body wt, g	471	473	461	454
		Liver wt, g	16.14	15.43	16.18	17.58*
		Liver wt, % body wt	3.44	3.27	3.51	3.87**
		Thyroid wt, g	0.020	0.020	0.021	0.021
		Thyroid wt, % body wt	0.0043	0.0043	0.0045	0.0045
		Thymus wt, g	0.350	0.336	0.337	0.293*
		Thymus wt, % body wt	0.0741	0.0716	0.0729	0.0645
F2	PND 21	Terminal body wt, g	49.9	50.3	49.9	48.2
		Liver wt, g	2.245	2.410	2.454*	2.473*
		Liver wt, % body wt	4.498	4.787*	4.889*	5.127**
		Thyroid wt, g	0.0060	0.0061	0.0068	0.0061
		Thyroid wt, % body wt	0.0122	0.0121	0.0137	0.0127
		Thymus wt, g	0.211	0.217	0.211	0.203
		Thymus wt, % body wt	0.421	0.434	0.425	0.420

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

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Table 5.6.1/01-8: Mean terminal body weight and absolute and relative weights of selected organs in female rats in the 2-generation reproduction study conducted with BCS-CN88460

			BCS-CN88460, dietary conc. in ppm			
Gen	**Phase**	**Parameter**	**0**	**150**	**450**	**1200**
F0	PPD 28	Terminal body wt, g	250	251	249	245
		Liver wt, g	10.46	10.68	11.34**	12.33**
		Liver wt, % body wt	4.19	4.27	4.55**	5.04**
		Thyroid wt, g	0.017	0.016	0.016	0.017
		Thyroid wt, % body wt	0.0067	0.0065	0.0063	0.0068
		Thymus wt, g	0.257	0.250	0.243	0.222*
		Thymus wt, % body wt	0.1030	0.1002	0.0971	0.0906**
F1	PND 21	Terminal body wt, g	46.7	46.8	47.2	48.5
		Liver wt, g	2.031	2.210*	2.333**	2.571**
		Liver wt, % body wt	4.417	4.709*	4.936**	5.293**
		Thyroid wt, g	0.0058	0.0057	0.0059	0.0060
		Thyroid wt, % body wt	0.0124	0.0123	0.0126	0.0124
		Thymus wt, g	0.210	0.217	0.215	0.217
		Thymus wt, % body wt	0.450	0.464	0.453	0.447
	PPD 28	Terminal body wt, g	268	270	265	262
		Liver wt, g	11.76	11.97	12.60*	13.02**
		Liver wt, % body wt	4.39	4.44	4.76**	4.97**
		Thyroid wt, g	0.017	0.016	0.017	0.017
		Thyroid wt, % body wt	0.0062	0.0059	0.0062	0.0066
		Thymus wt, g	0.274	0.281	0.282	0.254
		Thymus wt, % body wt	0.102	0.104	0.107	0.097
F2	PND 21	Terminal body wt, g	47.2	48.5	47.3	46.4
		Liver wt, g	2.181	2.401*	2.314*	2.382*
		Liver wt, % body wt	4.617	4.933*	4.873*	5.126**
		Thyroid wt, g	0.0060	0.0064	0.0064	0.0062
		Thyroid wt, % body wt	0.0128	0.0134	0.0136	0.0135
		Thymus wt, g	0.199	0.219	0.214	0.212
		Thymus wt, % body wt	0.421	0.454	0.451	0.455

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

2. Macroscopic findings

There were no treatment-related findings in the F0 males or females. In the F1 offspring culled on postpartum day 21, there was a slight increase in litters with either unilateral or bilateral dilated renal pelvis at 1200/600 ppm. This observation was not seen in the F2 offspring.

3. Microscopic findings

There were no treatment-related findings in the F0 males or females.

H. DEFICIENCIES

The study as described was compliant with the technical requirements of the relevant guidelines. None the less, dose selection was deficient as no adverse findings were noted.

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III. CONCLUSIONS

Based on the results of this 2-generation reproduction study, BCS-CN88460 was well-tolerated by both parents and offspring.

The No Observed Adverse Effect Level (NOAEL) for reproductive performance of the F0 and F1 adults, and the NOAEL for the survival, growth, and development of the F1 and F2 offspring, is considered to be 1200 ppm for adult males and 1200/600 ppm for adult females and offspring.

CA 5.6.2 Developmental toxicity studies

Report:	KCA 5.6.2/01; [redacted]; 2017; M-602126-01-1
Title:	BCS-CN88460 - Developmental toxicity study in the rat by gavage
Report No.:	SA 14192
Document No.:	M-602126-01-1
Guideline(s):	OECD guideline 414 (January, 2001) EEC Directive 2004/73/EC, Method B.31 (April, 2004) US EPA OCSPP Guideline number 870.3700 MAFF in Japan notification 12 Nousan N°8147 (November, 2000)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

BCS-CN88460 was administered to groups of 23 sperm-positive female Sprague Dawley rats by oral gavage from gestation day (GD) 6 to GD 20. The sperm-positive day was GD 0. The doses given were 0, 25, 125, and 625 mg/kg bw/day, with the test item suspended in aqueous 0.5% methylcellulose 400. The volume of administration was 10 mL/kg bw/day based on the most recent body weight recorded.

Clinical observations were recorded daily. Maternal body weights were recorded for all females on GD 0, 6, 10, 12, 14, 16, 18, and 21. Food consumption was also measured for all the females during the intervals GD 1-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, and 18-21. At scheduled sacrifice on GD 21, a blood sample was collected from 10 pregnant rats for clinical chemistry determinations and from 5 pregnant rats for bioanalytical examination, a macroscopic examination of the visceral organs was performed on all animals, the gravid uterine weight was recorded and all dams were evaluated for the number of corpora lutea, and number and status of implantations (resorptions and dead or live fetuses). In addition, the liver and thyroid gland from all females were weighed at scheduled sacrifice and were retained in 10% neutral buffered formalin. Histological examination was performed on the liver and thyroid gland from the first 10 pregnant dams in the control, mid, and high dose groups. Live fetuses were removed from the uterus, counted, weighed, sexed, and examined externally. Approximately half of the live fetuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half of the fetuses were eviscerated, skinned, fixed in absolute ethanol, and stained according to a modification of the Tyl and Marr technique for skeletal examination of bone and cartilage.

At least 21 females were pregnant in all groups including the control out of 23 mated females per group. Due to one accidental death at 25 mg/kg bw/day, there were in total 21, 21, 23, and 23 dams with viable fetuses at the end of the treatment period at 0, 25, 125, and 625 mg/kg bw/day, respectively.

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Up to the highest dose level tested of 625 mg/kg bw/day, there were no treatment-related maternal mortalities or clinical signs throughout the study.

At necropsy, the bioanalytical examination showed that the mean plasma concentration of BCS-CN88460 was below or marginally above the limit of quantification of 0.01 mg/L at the three dose levels tested, while there was essentially a dose-related increase in the mean plasma concentrations of the metabolites BCS-CX99798 and BCS-CX99799.

At 625 mg/kg bw/day
In dams, mean body weight gain between GD 6 and GD 8 was decreased by 43% compared to controls. Thereafter, throughout other study intervals, mean body weight gain was unaffected by treatment. Overall between gestation days 6 and 21, mean body weight gain was 5% lower than controls. Maternal corrected body weight change (body weight gain between GD0 and GD21 independent of the gravid uterus weight recorded at cesarean section) was also slightly lowered by 10% compared to controls. Mean food consumption was slightly decreased by 12% between gestation days 6 and 8 compared to controls. Thereafter, food consumption was similar to controls. At scheduled necropsy, the clinical chemistry determination revealed a lower mean bilirubin concentration and a lower mean alkaline phosphatase activity compared to controls. Mean absolute liver weight was 44% higher than in controls. At macroscopic examination, enlarged liver was noted in 14/23 females. At microscopic examination, minimal to slight hepatocellular centrilobular hypertrophy was noted in the liver of all dams checked (10/10) and minimal follicular cell hypertrophy was noted in 1/10 dams.

At cesarean section, the only treatment-related change consisted of a lower mean fetal body weight compared to controls. Fetal evaluation revealed no treatment-related malformations. At the external fetal examination, there were no treatment-related variations. The visceral fetal examination revealed an incresaed incidence of "bladder distended" and "renal pelvis (uni/bi): dilated (< severe)" at the fetal and litter levels, compared to controls. The skeletal fetal examination revealed an overall increase in delayed ossification, suggestive of a moderate delay in the fetal development. Treatment-related skeletal fetal changes consisted of an increased incidence of spontaneous variations at the fetal and litter levels in terms of incomplete ossification of "at least one bone of zygomatic arch (uni/bi)", "squamosal (uni/bi)", "hyoid centrum", "femur" and "humerus".

At 125 mg/kg bw/day
In dams up to necropsy, there were no treatment-related effects. At scheduled necropsy, the clinical chemistry determination revealed a 67% lower mean bilirubin concentration compared to controls. This change was considered to be treatment-related but not to be adverse as it was seen in isolation and does not represent any functional impairment in the animal. Mean absolute liver and thyroid gland weights were unaffected by treatment. There were no treatment-related macroscopic or microscopic changes.

At cesarean section, there were no treatment-related effects.

Fetal evaluation revealed no treatment-related malformations and no treatment-related variations at the external and visceral fetal examinations. The skeletal fetal examination revealed an increased incidence of 3 spontaneous variations at the fetal and litter levels in terms of incomplete ossification of "squamosal (uni/bi)", "hyoid centrum", and "humerus". As the only findings were restricted to a delay in ossification of commonly seen spontaneous variations and as the increased incidences at the fetal and litter levels were not statistically significantly different from the controls (only slightly above the range of in-house HCD), they were considered to represent only a slight and transient delay in fetal development with no adverse long-term consequences.

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At 25 mg/kg bw/day
In dams up to necropsy, there was no treatment-related adverese effect. At scheduled necropsy, the clinical chemistry determination revealed a 73% lower mean bilirubin concentration compared to controls. This change was considered to be treatment-related but not to be adverse as it was seen in isolation and does not represent any functional impairment in the test organism.

At cesarean section, there were no treatment-related effects.

Fetal evaluation revealed no treatment-related malformations or variations at the external, visceral, and skeletal fetal examinations.

In conclusion, a dose level of 625 mg/kg bw/day BCS-CN88460 administered to the pregnant Sprague Dawley rat by oral gavage was considered to be a Lowest Observed Adverse Effect Level (LOAEL) in terms of maternal and fetal toxicity. A dose level of 125 mg/kg bw/day was considered to be a No Observed Adverse Effect Level (NOAEL) in terms of maternal and fetal toxicity, based on the isolated clinical chemistry change seen in dams and the slight retarded ossification noted for a few elements of the skeleton in fetuses. A dose level of 25 mg/kg bw/day was considered to be a No Observed Effect Level (NOEL) in terms of maternal and fetal toxicity.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% w/w
CAS #	1255734-28-1
Stability of test compound:	Until 18 December 2014

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

3. Test animals:	
Species:	Rat
Strain:	Crl:CD (SD) Sprague-Dawley
Age:	11-13 weeks old
Weight at dosing:	
Source:	[redacted]
Acclimation period:	At least 11 days prior to start of treatment
Diet:	A04C-10 pelleted rodent diet from [redacted] *ad libitum*
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*
Housing:	Individual housing in suspended polycarbonate cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	Target of 10-15/hour
Photoperiod:	12 hours dark / 12 hours light

B. STUDY DESIGN

1. In life dates: 27 October 2014-27 November 2014

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2. Animal assignment and treatment

One hundred and twenty nulliparous female rats were obtained from the supplier. After the acclimation period, females were mated on a one-to-one basis with stock males of the same strain from the same supplier. Each morning following pairing, those female rats showing spermatozoa in a vaginal smear or a sperm plug in situ were considered as pregnant animals, and the day where evidence of mating was found was designated as gestation day 0 (GD0). Females were assigned to control and treated groups using a computerized randomization procedure (Pristima, version 6.3.2 build 17, Xybion Corp.) for each day of pairing. If possible, those females which were paired with the same male were not allocated to the same treatment group. Body weight means were checked after the mating period to ensure similar means among all groups.

3. Dose selection rationale

The doses administered in this study were selected based on the results of a range-finding study (SA 11321; M-598295-01-1, available upon request), where pregnant rats received BCS-CN88460 from GD6 through GD 20 at doses of 0, 70, 250, or 700 mg/kg bw/day. In that study,

- At 700 mg/kg bw/day,
  - mean maternal body weight gain was decreased by 70% between GD6 and GD8, although this change was not statistically significant;
  - overall mean maternal body weight gain between GD6 and GD21 was marginally but not statistically significantly reduced by 6% compared to controls;
  - at necropsy, enlarged liver was noted for all dams, and mean liver weight was increased by 87% compared to controls (statistically significant at $p \leq 0.01$);
  - no treatment-related changes were noted for litter parameters or at fetal external examination.
- At 250 mg/kg bw/day,
  - there were no effects on maternal body weight or body weight gain;
  - the incidence of enlarged liver was increased relative to controls (7/8 treated animals, against 1/7 in the controls), and liver weight was increased by 39% compared to controls (statistically significant at $p \leq 0.01$);
  - to treatment-related changes were noted for litter parameters or at fetal external examination.
- At 70 mg/kg bw/day,
  - there were no effects on maternal body weight or body weight gain;
  - the incidence of enlarged liver was increased compared to controls (4/6 treated animals, compared to 1/7 in controls) and liver weight was increased by 22% compared to controls (not statistically significant);
  - no treatment-related changes were noted for litter parameters or at fetal external examination.

Based on these results, the dose levels selected for the definitive developmental toxicity study were 0, 25, 125, and 625 mg/kg bw/day. The high dose of 625 mg/kg bw/day was expected not to exceed a Maximum Tolerated Dose (MTD) in the dams, the low dose of 25 mg/kg bw/day was expected to be a No Observed (Adverse) Effect Level for both maternal and fetal toxicity, and the mid dose of 125 mg/kg bw/day was chosen as it provided a 5-fold factor between the high and low doses.

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4. Dose preparation and analysis

The appropriate amount of test item was suspended on a weight / weight basis in an aqueous solution of 0.5% methylcellulose 400 and stored in air-tight bottles at approximately 5°C. Test formulations were prepared twice during the study.

Stability of the test item in 0.5% aqueous methylcellulose 400 was demonstrated previously at concentrations of 0.5 and 150 g/L for at least 27 days under similar conditions of usage and storage to those of the current study.

Homogeneity at the top, bottom, and middle of the suspensions was checked on the first formulation for the lowest (2.5 g/L) and the highest (62.5 g/L) concentrations. The mean values obtained from the homogeneity check were used as measured concentrations. In addition, the intermediate concentration of the first formulation and all concentrations of the second formulation were checked.

The homogeneity and concentration analyses provided results of 98-104% of nominal concentrations, which were within the in-house target range of 80-110% of nominal concentrations. The dose preparations were therefore considered acceptable for use.

5. Dose administration

The test item was administered once daily, from GD6 to GD20 inclusive, by oral gavage at a volume of 10ml/kg bw/day. The dose volumes were calculated on the basis of the animal's most recently recorded body weight. Control animals received an equivalent volume of vehicle.

The suspensions were mixed continuously before and during treatment with an electromagnetic stirrer. They were stored at approximately 5°C when not in use.

6. Statistics

Mean and standard deviation for all maternal, litter, and fetal parameters were calculated for each group. Statistical analyses were performed initially for all pregnant females which survived until the scheduled sacrifice. Where relevant, statistical analyses were conducted for all pregnant females with live fetuses only, or including pregnant females that did not survive until the scheduled sacrifice.

For body weight changes (calculated according to intervals); calculated corrected body weight change; carcass, liver, thyroid gland, and uterus weights; and clinical chemistry parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For food consumption calculated according to intervals, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was

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significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.

For the litter-based endpoints of number of corpora lutea, number of implantation sites, number of resorptions (early, late, and total), and pre- and post-implantation loss percentages, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For fetal body weight (both combined sexes and per sex), mean and standard deviation were calculated for each group. The Levene test was performed to compare the homogeneity of group variances. If the Levene test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Levene test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Levene test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Levene test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.

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If one or more group variances were equal to 0, means were compared using non-parametric procedures.

C. METHODS

1. Observations

All cages were checked for moribund or dead animals twice daily, once in the morning and again in the afternoon, except on weekends, when these checks were conducted only once daily. All clinical signs were recorded for individual animals, and all animals were examined daily for clinical signs from GD0 to GD21.

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

Full and empty feeder weights were recorded on gestation days 1 (full feeder weight only), 6, 8, 10, 12, 14, 16, 18, and 21 (empty feeder weights only), and food spillage was also noted. From these records, mean daily food consumption was calculated.

On the day of scheduled sacrifice, blood samples were taken from ten pregnant animals in each group by aorta puncture. Animals were not diet fasted overnight prior to bleeding. Animals were anesthetized by isoflurane inhalation, and blood was collected on clot activator in order to conduct serum clinical chemistry. Serum was separated and samples were frozen at -20°C. Any significant change in the general appearance of the serum was recorded.

Blood samples were taken from an additional five pregnant rats in each group at necropsy, for determination of the concentrations of BCS-CN88460 and its major metabolites ‘798 and ‘799. Blood was collected from the aorta into heparinized vials and plasma was prepared by centrifugation, then stored in the dark at approximately -20°C for shipment with dry ice.

2. Post mortem examinations

Animals found dead were subjected to a macroscopic examination of the visceral organs. The number of implantations and corpora lutea were noted when present. In the case of no visible uterine implants, uterine horn(s) were immersed in a 10% solution of ammonium sulfide according to the Salewski method, in order to visualize any sites which were not apparent. Live fetuses, if any, were killed by injection of Dolethal®, and the tissues and carcass of the dams and fetuses were discarded.

On GD21, all surviving females were anesthetized with isoflurane inhalation and then euthanized by exsanguination. Each female was subjected to macroscopic examination of the visceral organs. The liver of all females was weighed fresh and the uterine content was examined. The liver and thyroid gland (with parathyroid) were retained in 10% neutral buffered formalin from all females, and the liver and thyroid gland from the first 10 pregnant females in all groups were processed and embedded in paraffin wax, and histological slides were prepared and stained with hematoxylin and eosin. Histopathology on the liver and thyroid gland was conducted for the first 10 pregnant females in the control, mid and high dose groups.

The reproductive tract was weighed (gravid uterine weight) and dissected out, and the following parameters were recorded without knowledge of the treatment group:

- Number of corpora lutea
- Number of implantations
- Number of resorptions, classified as early or late
- Number of live and dead fetuses
- Sex of live fetuses
- Individual weights of live fetuses

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Uterine horn(s) without visible implantations were immersed in a 10% solution of ammonium sulfide according to the Salewski method in order to visualize any sites which were not apparent. The tissues and carcass of the dams were discarded without further examination. Intra-uterine death was classified according to Gleich and Frohberg as

- Early resorptions: presence of implantation site(s), or macroscopic discrimination between fetal residues and placental material not possible;
- Late resorptions: distinct macroscopic discrimination between fetal and placental remains possible.

Dead fetuses were those fetuses which were dead at maternal necropsy but which showed distinct digits on fore- and hind-paws.

3. Fetal examinations

All fetal examinations were recorded without knowledge of treatment group.

All live fetuses were killed by subcutaneous injection (0.02 ml/fetus) of Dolethal® and subjected to an external examination. Approximately half of the live fetuses from each litter were immersed in Bouin's fluid for subsequent internal examination following free-hand sectioning. The remaining half were skinned, eviscerated, and then placed in absolute ethanol before staining with alizarin red S and alcian blue according to a modified staining technique, for skeletal examination of bone and cartilage. All specimens were archived.

Structural deviations were classified as follows:

- Malformation: permanent structural change that is likely to adversely affect survival or health;
- Variation: change that occur within the normal population under investigation and is unlikely to adversely affect survival or health; this might include a delay in growth or morphogenesis that has otherwise followed a normal pattern of development.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no treatment-related mortalities in any group. One animal at 25 mg/kg bw/day was found dead on study day 18, with congested red lungs and foamy content in the trachea noted at necropsy. The cause of death was attributed to a gavage error and was thus unrelated to the administration of the test item.

B. CLINICAL OBSERVATIONS

There were no treatment-related clinical signs in any group throughout the period of the study. Hair loss was noted in all groups including controls at various points throughout the study, and one animal in the 625 mg/kg bw/day dose group was noted with piloerection on one occasion.

C. BODY WEIGHT

There was no treatment-related effect on body weight or body weight gain at either 25 or 125 mg/kg bw/day. At 625 mg/kg bw/day, there was a non-statistically significant decrease in body weight gain between gestation days 6 and 8, and a slight but statistically non-significant decrease in overall body weight gain (gestation days 6-21), and in corrected body weight change.

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Table 5.6.2/01-1: Maternal body weight during gestation after oral gavage administration of BCS-CN88460

		BCS-CN88460, dose in mg/kg bw/day			
Gestation day		**0**	**25**	**125**	**625**
0	g	269.4	265.6	269.2	270.2
	% control		98.6	99.9	100.3
6	g	313.3	310.0	311.3	311.4
	% control		98.9	99.4	99.4
8	g	317.9	314.7	316.7	314.0
	% control		99.0	99.6	98.8
10	g	329.5	323.4	329.9	326.9
	% control		98.1	100.1	99.2
12	g	341.8	339.1	341.2	338.3
	% control		99.2	99.8	99.0
14	g	353.3	350.5	352.7	348.3
	% control		99.2	99.8	98.6
16	g	372.5	368.8	371.8	366.6
	% control		99.0	99.8	98.4
18	g	399.0	397.6	398.9	393.2
	% control		99.6	100.0	98.5
21	g	457.1	453.0	457.3	448.2
	% control		99.1	100.0	98.1

Table 5.6.2/01-2: Maternal body weight change during gestation after oral gavage administration of BCS-CN88460

		BCS-CN88460, dose in mg/kg bw/day			
Gestation days		**0**	**25**	**125**	**625**
0-6	g	43.9	44.4	42.1	41.2
	% control		101.1	95.9	93.8
6-8	g	4.6	4.7	5.4	2.6
	% control		102.2	117.4	56.5
8-10	g	11.6	8.7	13.2	12.9
	% control		75.0	113.8	111.2
10-14	g	23.8	27.1	22.8	21.4
	% control		113.9	95.8	89.9
14-18	g	45.7	47.1	46.2	44.9
	% control		103.1	101.1	98.2
18-21	g	58.1	55.4	58.4	55.0
	% control		95.4	100.5	94.7
6-21	g	143.8	143.0	146.0	136.8
	% control		99.4	101.5	95.1

D. FOOD CONSUMPTION

At 25 and 125 mg/kg bw/day, there was no effect on food consumption at any period during the study. At 625 mg/kg bw/day, there was a slight but statistically significant decrease in food consumption on between gestation days 6 and 8.

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Table 5.6.2/01-3: Maternal food consumption, in g/day, during gestation after oral gavage administration of BCS-CN88460

		BCS-CN88460, dose in mg/kg bw/day			
Gestation days		**0**	**25**	**125**	**625**
1-6	g/day	26.1	25.7	26.7	25.3
	% control		98.5	102.6	96.9
6-8	g/day	26.4	26.1	26.6	23.1*
	% control		99.0	100.9	87.6
8-10	g/day	27.7	27.1	28.4	27.4
	% control		97.6	102.5	98.9
10-12	g/day	27.6	28.7	28.3	27.1
	% control		104.2	102.8	98.3
12-14	g/day	28.9	29.1	30.0	27.5
	% control		100.9	103.8	95.4
14-16	g/day	30.0	29.1	29.9	29.4
	% control		97.0	99.8	98.0
16-18	g/day	29.5	30.4	31.0	30.9
	% control		103.1	105.0	104.6
18-21	g/day	30.7	30.8	32.1	30.3
	% control		100.2	104.5	98.5

E. CLINICAL CHEMISTRY AND BIOANALYSIS

There was a statistically significant treatment-related decrease in total bilirubin concentration at all doses. This is not considered an adverse effect, but as an indicator of biological effect of the test item.

Table 5.6.2/01-4: Mean maternal bilirubin concentration during gestation after oral gavage administration of BCS-CN88460

	BCS-CN88460, dose in mg/kg bw/day			
	0	**25**	**125**	**625**
Total bilirubin umol/L	0.51	0.24	0.17**	0.08***

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

The plasma concentration of BCS-CN88460 and its metabolites BCS-CX99798 and BCS-CX99799 were measured at the end of the study in five animals from each group. The concentration of the test item itself was only marginally above the limit of quantification at all doses, although there was a dose-related increase in the concentrations of the two metabolites, indicating that the test item had been extensively absorbed and metabolized after administration.

Table 5.6.2/01-5: Mean concentrations of test item BCS-CN88460 and the metabolites BCS-CX99798 and BCS-CX99799 in plasma at necropsy

	BCS-CN88460, dose in mg/kg bw/day			
	0	**25**	**125**	**625**
BCS-CN88460	< LOQ	< 0.012	0.015 ± .007	0.017 ± 0.003
BCS-CX99798	< LOQ	0.039 ± 0.020	0.147 ± 0.037	0.676 ± 0.260
BCS-CX99799	< LOQ	0.339 ± 0.197	0.273 ± 0.106	0.607 ± 0.306

F. NECROPSY

There were no effects on maternal carcass weight, or on the weight of either liver or thyroid, at 25 or 125 mg/kg bw/day. At 625 mg/kg bw/day, maternal carcass weight was slightly but statistically non-significantly decreased. Liver weight in this group was statistically significantly increased, along with an increase in the incidence of enlarged liver at macroscopic

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examination and in the incidence of hepatocellular hypertrophy. Also at 625 mg/kg bw/day, thyroid weight was not altered but one female showed minimal follicular cell hypertrophy.

Table 5.6.2/01-6: Mean maternal carcass weight, liver weight, and thyroid weight after oral gavage administration of BCS-CN88460

		BCS-CN88460, dose in mg/kg bw/day			
		0	**25**	**125**	**625**
Maternal carcass weight	g	348.5	344.6	348.9	341.3
	% control		98.9	100.1	97.9
Liver wt	g	14.1	14.0	15.5	20.3
	% control		99.1	109.8	143.8
Macroscopy: N examined		21	21	23	23
Liver, enlarged		0	0	0	14
Microscopy: N examined		10	0	10	10
Hepatocellular hypertrophy, centrilobular, diffuse					
Minimal		0		0	5
Slight		0		0	5
Total		0		0	10
Thyroid wt	g	0.0166	0.0154	0.0164	0.0170
	% control		92.8	98.5	102.2
Microscopy: N examined		10	0	10	10
Follicular cell hypertrophy, diffuse					
Minimal		0		0	1
Total		0		0	1

G. CAESAREAN SECTION DATA

There was no effect of treatment with BCS-CN88460 on pregnancy rate, number of live fetuses, number of implant sites per dam, pre- or post-implantation losses, early and late resorptions, fetal death status, or sex distribution of fetuses. Fetal body weight was slightly decreased at 625 mg/kg bw/day, this decrease was only statistically significant for female fetuses.

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Table 5.6.2/01-7: Caesarean section data from dams administered BCS-CN88460 by oral gavage during gestation

	BCS-CN88460, dose in mg/kg bw/day				Hist. Control
Maternal data	**0**	**25**	**125**	**625**	**Data**
No. Animals assigned	23	23	23	23	NA
No. animals pregnant	21	22	21	21	NA
Pregnancy rate, %	91	96	100	100	NA
No. Animals non-pregnant	2	1	0	0	NA
Maternal wastage:					NA
Intercurrent death or sacrifice, total	0	1	0	0	NA
Intercurrent death or sacrifice, pregnant	0	1	0	0	NA
Premature delivery	0	0	0	0	NA
Intercurrent death or sacrifice, non-pregnant	0	0	0	0	NA
Uterine data at scheduled sacrifice					NA
Total no. corpora lutea	382	367	410	415	NA
Corpora lutea per dam	18.2	17.5	17.8	18.0	15.74-18.39
Total no. implantations	328	320	352	369	NA
Implantations per dam	15.6	15.2	15.3	16.0	14.38-16.04
Total no. litters	21	21	23	23	NA
Total no. live fetuses	307	306	333	343	NA
Live fetuses per dam	14.6	14.6	14.5	14.9	13.52-15.17
Total no. dead fetuses	0	0	0	0	NA
Total no. early resorptions	21	13	15	25	NA
Early resorptions per dam	1.0	0.6	0.7	1.1	0.565-1.391
Total no. late resorptions	0	1	4	1	NA
Late resorptions per dam	0	0	0.2	0	0.0-0.217
Litters with total resorptions	0	0	0	0	NA
Mean fetal wt, g, combined sexes	5.59	5.63	5.63	5.37	5.36-5.61
Mean fetal wt, g, males	5.70	5.75	5.80	5.51	5.52-5.75
Mean fetal wt, g, females	5.47	5.48	5.49	5.21*	5.21-5.48
Sex ratio, % males	51.1	56.9	46.8	53.4	NA
Sex ratio, % males per litter	51.0	56.3	47.4	53.9	46.5-53.5
Pre-implantation loss per dam, %	13.33	12.31	13.81	10.75	3.32-15.43
Post-implantation loss per dam, %	6.34	4.32	5.25	7.10	3.95-10.37

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

H. FETAL FINDINGS

The incidence of fetal malformations was not affected by treatment. A total of eight fetuses were observed with malformations at external, visceral, or skeletal examination. However, as malformations were seen in all groups including control and as there were no commonalities among the affected fetuses, these are not considered to be related to treatment.

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Table 5.6.2/01-8: Summary of fetal malformations

mg/kg bw/day	Dam	Fetus	External	Visceral	Skeletal
0	1F3750	15	No malformation	Not examined	6th sternebra: branched with branched cartilage. Xyphoid process: split
25	2F3762	8	No malformation	Situs inversus: total	Not exam.
	2F3768	8	No malformation	Retina (uni/bi): fold	Not exam.
		10	No malformation	Retina (uni/bi): fold	Not exam.
125	3F3800	3	No malformation	Not examined	Anterior and posterior fontanelles: enlarged. 6th and 7th cervical arch (uni): fused with fused cartilage. 1st and 2nd costal cartilage (uni): fused. 5th costal cartilage uni): discontinuous and misshapen. 1st sternebra: bipartite. 2nd sternebra: incomplete ossification. 5th sternebra: unossified. 14th thoracic rib (bi) with long cartilage. 7th costal cartilage (bi): not attached to sternum. 27 presacral vertebrae. 4th thoracic centrum: incomplete ossification.
625	4F3810	10	Trunk: short (severe) Anus: absent Tail: thread-like (entire length) Forepaw (bi): malrotated outward	Testis(bi): malpositioned (cranial region) Adrenal glands (bi): absent Kidneys: fused Renal pelvis (uni): dilated (< severe) Intestines: colon distended Anus narrowed	Not examined
	4F3802	2	No malformation	Retina(uni/bi): fold	Not examined
	4F3803	8	No malformation	Retina(uni/bi): fold	Not examined

There was no effect on the incidence of external fetal variations in any dose group. The finding "tail, bent" was noted with very low incidence and for litter incidence did not exceed the in-house historical control data.

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Table 5.6.2/01-9: Incidence of external fetal variations

		BCS-CN88460, dose in mg/kg bw/day				HCD, %
		0	25	125	625	
N examined	Litters	21	21	23	23	
	Fetuses	307	306	333	343	
Tail, bent	Litters	0	1	2	1	
	%	0.0	4.8	8.7	4.3	0.0-4.3
	Fetuses	0	1	2	2	
	%	0.0	0.3	0.6	0.6	0.0-0.3

There was no increase in the incidence of fetal visceral findings at 25 or 125 mg/kg bw/day. At 625 mg/kg bw/day, there was a treatment-related, statistically significant increase in the incidence of "bladder, distended", and of "renal pelvis (unilateral / bilateral) dilated (< severe)" which exceeded the historical control data. Although the incidence of "thymic remnant present (unilateral / bilateral)" was increased in a dose-related manner, it was within the historical control data and is therefore considered to be not related to treatment.

Table 5.6.2/01-10: Incidence of fetal visceral variations

		BCS-CN88460, dose in mg/kg bw/day				HCD, %
		0	25	125	625	
N examined	Litters	21	21	23	23	
	Fetuses	148	148	159	167	
Thymic remnant present (uni/bi)	Litters	3	4	6	8	
	%	14.3	19.0	26.1	34.8	8.7-40.9
	Fetuses	3	6	7	12*	
	%	2.0	4.1	4.4	7.2	2.5-10.3
Bladder, distended	Litters	0	0	0	1	
	%	0	0	0	4.3	0.0-0.0
	Fetuses	0	0	0	3	
	%	0	0	0	1.8	0.0-0.0
Renal pelvis (uni/bi): dilated (< severe)	Litters	0	1	2	6*	
	%	0.0	4.8	8.7	26.1	0.0-21.7
	Fetuses	0	3	2	7*	
	%	0.0	2.0	1.3	4.2	0.0-3.2

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

There was no increase in the incidence of fetal skeletal findings at 25 mg/kg bw/day. At 125 mg/kg bw/day, there was a slight delay in ossification, however as the findings in this group were commonly seen variations and the increased incidence only slightly exceeded the in-house historical control data and was not statistically significant for any of the findings, these are considered to represent only a slight, transient delay in fetal development with no adverse consequences. At 625 mg/kg bw/day, there was a moderate delay in ossification of some structures, which exceeded the in-house historical control data and / or was statistically significant.

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Table 5.6.2/01-11.: Incidence of fetal skeletal variations

		BCS-CN88460, dose in mg/kg bw/day				HCD, %
		0	25	125	625	
N examined	Litters	21	21	23	23	
	Fetuses	159	158	174	176	
At least one bone of zygomatic arch (uni/bi) incomplete ossification	Litters	3	3	7	12*	
	%	14.3	14.3	30.4	52.2	0.0-34.8
	Fetuses	4	6	10	15*	
	%	2.5	3.8	5.7	8.5	0.0-8.8
Squamosal: incomplete ossification (uni/bi)	Litters	1	1	3	4	
	%	4.8	4.8	13.0	17.4	0.0-9.1
	Fetuses	1	2	3	4	
	%	0.6	1.3	1.7	2.3	0.0-1.2
Hyoid centrum: incomplete ossification	Litters	4	2	8	9	
	%	19.0	9.5	34.8	39.1	0.0-26.1
	Fetuses	4	2	11	14*	
	%	2.5	1.3	6.3	8.0	0.0-7.0
Femur: incomplete ossification (uni/bi)	Litters	2	4	5	5	
	%	9.5	19.0	21.7	21.7	0.0-26.1
	Fetuses	2	4	8	15**	
	%	1.3	2.5	4.6	8.5	0.0-6.4
Humerus: incomplete ossification (uni/bi)	Litters	0	0	3	2	
	%	0.0	0.0	13.0	8.7	0.0-4.3
	Fetuses	0	0	4	3	
	%	0.0	0.0	2.3	1.7	0.0-0.6

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Other skeletal findings which were noted to have increased were not statistically significant, did not exceed or only marginally exceeded the in-house historical control data, and / or had no relationship to dose, and were thus considered to be incidental and not related to administration of BCS-CN88460 during gestation.

I. DEFICIENCIES

Food intake was recorded from gestation day 1 instead of gestation day 0, as stated in the JMAFF guideline (November 2000).

III. CONCLUSIONS

Based on isolated clinical chemistry changes seen in dams and the slight retarded ossification noted for a few elements of the skeleton in fetuses, the dose level of 125 mg/kg bw/day was considered to be the No Observed Adverse Effect Level for both maternal and fetal toxicity. The dose level of 25 mg/kg bw/day was the No Observed Effect Level for both maternal and fetal toxicity.

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Report:	KCA 5.6.2/02; [redacted]; 2017; M-588469-01-1
Title:	BCS-CN88460 - Developmental toxicity study in the rabbit by gavage - Final report
Report No.:	SA 15122
Document No.:	M-588469-01-1
Guideline(s):	OECD guideline 414 (January, 2001); EEC Directive 2004/73/EC, Method B.31 (April, 2004); US EPA OCSPP Guideline number No 870.3700; MAFF IN JAPAN notification 12 Nousan No 8147, (November, 2000)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

BCS-CN88460 was administered to groups of 23 time-mated pregnant female New Zealand White rabbits by oral gavage from gestation day (GD) 6 to 28. The sperm-positive day was GD 0. The doses given were 0, 10, 70, and 500 mg/kg bw/day, with the test item suspended in aqueous solution of 0.5% methylcellulose 400. The volume of administration was 4 ml/kg based on the most recent body weight recorded.

Clinical observations were recorded daily from GD0, GD1, or GD2 to GD29. Maternal body weights were recorded for all females on GD 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Food consumption was also measured for all females during the intervals GD 3-4, 4-5, 5-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, 18-20, 20-22, 22-24, 24-26, 26-28, and 28-29. At scheduled sacrifice on GD 29, a macroscopic examination of the visceral organs was performed, the gravid uterine weight was recorded, and the dams were evaluated for number of corpora lutea, number and status of implantations (resorptions and dead or live fetuses). In addition, the liver was weighed at scheduled sacrifice for all pregnant females. Live fetuses were removed from the uterus, counted, weighed, sexed, and examined externally. The heads of the fetuses from approximately half of each litter were immersed in Davidson's fixative then in Bouin's fluid and the internal structures examined after fixation. The bodies of all fetuses were dissected for soft tissue anomalies and sex determination. Fetuses were eviscerated, skinned, and fixed in absolute ethanol before staining. A modification of the Staples and Schnell technique was applied and a subsequent skeletal examination was performed.

The pregnancy rate was 100% in all groups except in the 10 mg/kg bw/day group with a single non-pregnant female for the study.

<u>At 500 mg/kg bw/day</u>
There was a BCS-CN88460-related maternal toxicity characterized by at least one abortion, a statistically-significant mean body weight loss between GD 6 and 8 followed by a slight decrease in mean body weight gain at least between GD 8 and 10, a mean maternal corrected body weight change of -29% compared to the control group, a reduction of the mean food consumption between 14% and 22% between GD 6 and GD 14 attaining statistical significance during the GD 10-14 interval and an incresae in the mean liver weight of 16% versus the control group.

At cesarean section, no treatment-related changes were noted in the post-implantation data (mean number of early and late resorptions, percentage of post-implantation loss) and fetal data (mean litter size, mean fetal body weight, and percentage of males). No dead fetuses were observed.
There were no treatment-related external, visceral, or skeletal findings at the fetal examinations.

<u>At 70 and 10 mg/kg bw/day</u>
Except for a statistically non-significant decrease of 75% in mean body weight gain between GD 6 and 8 at 70 mg/kg bw/day, there were no treatment-related maternal effects.

There were no treatment-related changes in the post-implantation or fetal data.
There were no treatment-related external, visceral, or skeletal findings at fetal examinations.

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In conclusion, a dose level of 500 mg/kg bw/day BCS-CN88460 administered to the pregnant New Zealand White rabbit by oral gavage resulted in maternal toxicity as evidenced by at least one abortion, statistically significant body weight loss, reduced food consumption, lower mean maternal corrected body weight change, and increase in mean liver weight. Fetal development was unaffected by treatment at any dose level tested. A dose level of 70 mg/kg bw/day was considered to be a No Observed Adverse Effect Level (NOAEL) in terms of maternal toxicity, while a dose level of 500 mg/kg bw/day was considered to be a NOEL in terms of developmental toxicity.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-086492
Purity:	94.2% (w/w)
CAS #	1255734-28-1
Stability of test compound:	Until 28 April 2016

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

3. Test animals:	
Species:	rabbit
Strain:	New Zealand White Crl:KBL(NZW)
Age:	18 ± 1 weeks of age
Weight at dosing:	
Source:	[redacted]
Acclimation period:	At least four days prior to commencement of dosing
Diet:	110 C-10 pelleted animal diet from [redacted], France, ad libitum
Water:	Filtered and softened tap water from the municipal water supply, ad libitum
Housing:	Individual housing in suspended stainless steel wire mesh cages
Environmental conditions:	
Temperature:	17-21°C
Humidity:	40-70%
Air changes:	Target of 10-15/hour
Photoperiod:	16 hours light / 8 hours dark

B. STUDY DESIGN

1. In life dates: 27 October 2015-8 December 2015

2. Animal assignment and treatment

Nulliparous females were mated at the supplier with stock males of the same strain, and were received on gestation day 0, 1, or 2. The day of mating was designated as gestation day 0.

On each day of mating, the females were assigned to control and treated groups using a computerized randomization procedure (Pristima, version 7.0.0 build 22, Xybion Corp.). As much as possible, the same male was not mated with more than two females in each treatment group. Body weight means were checked before treatment to ensure similar means

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among all groups. Any animal showing signs of ill health prior to treatment initiation was removed from the study and replaced.

3. **Dose selection rationale**
In a preliminary range-finding study, pregnant rabbits were administered BCS-CN88460 by oral gavage at 0, 50, 150, and 450 mg/kg bw/day on gestation days 6 through 28 inclusive. In that study, there was no effect on maternal mortality, pregnancy rate, any litter parameters, or fetal body weight. Liver weight was increased by approximately 20-21% at all dose levels. There were no treatment-related findings at fetal external examination.

One female at 450 mg/kg bw/day had total resorption of the litter (2 early resorptions), however as it occurred in isolation it is considered as not treatment-related. Few or no feces were observed occasionally in most animals in this group. At the beginning of the treatment period (gestation days 6-8), there was a mean maternal body weight loss of 30g, compared to a gain of 30g in the control group. Otherwise, body weight parameters including corrected body weight change were unaffected by the treatment. Mean food consumption was statistically significantly decreased on gestation days 8-10, while decreases from gestation days 6-8 and gestation days 10-12 were not statistically significant.

It was considered that 450 mg/kg bw/day BCS-CN88460 caused slight maternal toxicity, but was an No Observed Effect Level for developmental toxicity in that study.
Based on these findings, the doses selected for this study were 0, 10, 70, and 500 mg/kg bw/day.

4. **Dose preparation and analysis**
The appropriate amount of test item was suspended in an aqueous solution of 0.5% methylcellulose 400 and stored at approximately 5°C. Test formulations were prepared six times during the study for all concentrations. In view of the analytical results on the fourth formulation of the lowest concentration at 2.5 g/L, an additional formulation was prepared and analyzed for this concentration.

Stability of the test item in 0.5% aqueous methylcellulose 400 was demonstrated in a previous study, at concentrations of 0.5 and 150 g/L for up to 27 days under similar conditions to those of the current study.

Homogeneity of the suspensions was checked on the first formulation for the lowest and the highest concentrations, 2.5 and 125 g/L. The mean values obtained from the homogeneity check were used as measured concentrations. In addition, the intermediate concentration of the first formulation and all concentrations of the remaining formulations were checked.

The homogeneity and concentration analyses showed results of 90-102% of nominal concentrations, which was within the in-house target range of 90-110% of nominal concentrations.

5. **Dose administration**
The test item was administered once daily by oral gavage, on gestation days 6 through 28 inclusive, at a volume of 4 ml/kg bw/day. Dosing was based on each animal's most recent recorded body weight. Control animals received an equivalent volume of 0.5% aqueous methylcellulose 400.

6. **Statistics**

Mean and standard deviation for all maternal, litter, and fetal parameters were calculated for each group. Statistical analyses were performed initially for all pregnant females which survived until the scheduled sacrifice. Where relevant, statistical analyses were conducted

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for all pregnant females with live fetuses only, or including pregnant females that did not survive until the scheduled sacrifice.

For body weight changes (calculated according to intervals); calculated corrected body weight change; carcass, liver, thyroid gland, and uterus weights; and clinical chemistry parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For food consumption calculated according to intervals, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.

For the litter-based endpoints of number of corpora lutea, number of implantation sites, number of resorptions (early, late, and total), and pre- and post-implantation loss percentages, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

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For fetal body weight (both combined sexes and per sex), mean and standard deviation were calculated for each group. The Levene test was performed to compare the homogeneity of group variances. If the Levene test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Levene test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Levene test on log-transformed data was not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Levene test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.
If one or more group variances were equal to 0, means were compared using non-parametric procedures.

C. METHODS

1. Observations

Animals were examined daily from arrival through gestation day 29. All cages were checked for dead or moribund animals, or animals showing signs of abortion, twice daily, once in the morning and again in the afternoon (except at weekends and public holidays when such checks were only carried out once daily).
Body weights were recorded on gestation days 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 29.
Full feeders were weighed on gestation days 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 28, while empty feeders were weighed on gestation days 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 29. Mean daily food consumption was calculated, and food spillage was also noted.

2. Post mortem examinations

Animals killed for human reasons were euthanized by intravenous injection of sodium pentobarbital. These animals were subjected to a macroscopic examination of the visceral organs. The number of corpora lutea and the number and type of implantations were noted when present. In cases where there were no visible uterine implants but corpora lutea were present, uterine horn(s) were immersed in a 20% solution of ammonium sulfide according to the Salewski method to visualize any sites which were not apparent. Live fetuses, if any, were killed by injection of sodium pentobarbital. The tissues and carcass of the dams were then discarded without further examination.

At scheduled sacrifice on gestation day 29, all surviving females were killed by intravenous injection of sodium pentobarbital. Each female was first subjected to a macroscopic examination of the visceral organs. The liver of all pregnant females was weighed fresh, and two central sections taken from the median and left lobes of the liver, as well as the thyroid

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gland from all females, were retained in 10% neutral buffered formalin for possible histological examination.

The reproductive tract was weighed (gravid uterine weight), dissected out, and the number of corpora lutea, implantations, early or late resorptions, and live or dead fetuses, as well as the individual weights of fetuses, were recorded without knowledge of treatment group.

In cases where there were no visible uterine implants but corpora lutea were present, uterine horn(s) were immersed in a 20% solution of ammonium sulfide according to the Salewski method, in order to visualize any implantation sites which were not apparent. Tissues and carcass of the dams were then discarded.

Intra-uterine resorption death was classified according to Gleich and Frohberg, as

- early resorptions: macroscopic discrimination between embryo-fetal residues and placental material was not possible.
- late resorptions: distinct macroscopic discrimination between embryo-fetal and placental tissues was possible.

Dead fetuses were defined as non-live fetuses at term with no degenerative changes.

On the day of scheduled sacrifice, a blood sample was collected from the ear of at least five pregnant rabbits from each group, just prior to necropsy and between 22 and 24 hours after the last dosing by gavage. Blood was collected into heparinized vials and plasma was prepared by centrifugation, then stored in the dark at approximately -20°C until shipment with dry ice for analysis to determine the plasma concentrations of the test item and its potential major metabolites.

3. Fetal examinations

Fetal examinations were conducted at final sacrifice only.

All live fetuses were killed by subcutaneous injection of sodium pentobarbital. All fetuses whether alive or dead at Caesarean section were subjected to an external examination. After internal examination of the neck, the heads of fetuses from approximately half of each litter was immersed in Davidson's fixative then in Bouin's fluid, and the internal structures were examined after fixation. The bodies of all fetuses were dissected for soft tissue abnormalities and sexed. The fetuses were then eviscerated, skinned, and fixed in absolute ethanol before staining by a modification of the Staples and Schnell staining technique.

Structural deviations were classified as:

- Malformation: a permanent structural change that is likely to adversely affect the survival or health of the animal.
- Variation: a change that occurs within the normal population under investigation, which is unlikely to adversely affect survival or health. This might include a delay in growth or morphogenesis that has otherwise followed a normal pattern of development.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities at 10 or 70 mg/kg bw/day. At 500 mg/kg bw/day, two dams aborted prior to the end of the study. One animal aborted on gestation day 23, with two corpora lutea, two implantation sites, and one early resorption. This animal lost 20 g between gestation day 6 and gestation day 8. At macroscopy, there were no observations. The presence of fewer than four implantation sites is likely to severely increase the risk of abortion in the rabbit. The body weight loss of this animal between gestation day 6 and gestation day 8 was similar to that of the

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rest of the group (19 g body weight lost). Based on these factors, this abortion is considered not related to treatment.

The other female aborted on gestation day 26, showing 13 corpora lutea, 11 implantation sites, and no early resorptions. This animal lost 170 g between gestation day 6 and gestation day 8. At macroscopic examination, all lobes of the liver were pale with marked, multiple diffuse white foci, and the spleen had multifocal depressions on the surface. It is likely that the abortion observed in this animal was indirectly related to treatment due to the poor health status of the animal.

B. CLINICAL OBSERVATIONS

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

C. BODY WEIGHT

There was no treatment-related effect on body weight or body weight gain at 10 mg/kg bw/day. Body weight change was decreased at 70 mg/kg bw/day between gestation days 6 and 8. At 500 mg/kg bw/day, body weight change was decreased at several intervals, and overall body weight gain between gestation days 6 and 29 was also decreased.

Table 5.6.2/02-1: Mean maternal body weight in rabbits administered BCS-CN88460 by oral gavage during gestation

		BCS-CN88460, dose in mg/kg bw/day			
Gestation day		**0**	**10**	**70**	**500**
3	kg	3.482	3.421	3.413	3.485
	% control		98.2	98.0	100.1
6	kg	3.493	3.475	3.477	3.533
	% control		99.5	99.5	101.1
8	kg	3.521	3.506	3.484	3.515
	% control		99.6	98.9	99.8
10	kg	3.573	3.541	3.516	3.539
	% control		99	98.4	99.0
12	kg	3.608	3.572	3.542	3.550
	% control		99.0	98.2	98.4
14	kg	3.649	3.623	3.588	3.595
	% control		99.3	98.3	98.5
16	kg	3.680	3.653	3.613	3.619
	% control		99.3	98.2	98.3
18	kg	3.707	3.672	3.633	3.640
	% control		99.1	98.0	98.2
20	kg	3.727	3.700	3.656	3.651
	% control		99.3	98.1	98.0
22	kg	3.751	3.737	3.680	3.688
	% control		99.6	98.1	98.3
24	kg	3.773	3.764	3.714	3.714
	% control		99.8	98.4	98.4
26	kg	3.794	3.794	3.744	3.723
	% control		100.0	98.7	98.1
29	kg	3.868	3.864	3.808	3.808
	% control		99.9	98.4	98.4

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Table 5.6.2/02-2: Mean maternal body weight change in rabbits administered BCS-CN88460 by oral gavage during gestation

		BCS-CN88460, dose in mg/kg bw/day			
Gestation day		**0**	**10**	**70**	**500**
6-8	kg	0.028	0.032	0.007	-0.019**
	% control		114.3	25.0	-67.9
8-10	kg	0.051	0.035	0.031	0.024
	% control		68.6	60.8	47.1
10-14	kg	0.076	0.082	0.073	0.057
	% control		107.9	96.1	75.0
14-18	kg	0.058	0.049	0.044	0.044
	% control		84.5	75.9	75.9
18-22	kg	0.044	0.065	0.047	0.048
	% control		147.7	106.8	109.1
22-26	kg	0.043	0.057	0.064	0.036
	% control		132.6	148.8	83.7
26-29	kg	0.074	0.070	0.063	0.085
	% control		94.6	85.1	114.9
6-29	kg	0.375	0.390	0.330	0.275
	% control		104.0	88.0	73.3

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

D. FOOD CONSUMPTION

At 10 and 70 mg/kg bw/day, there was no effect of BCS-CN88460 administration on food consumption. At 500 mg/kg bw/day, there was a decrease in food consumption between gestation days 6 and 8, between gestation days 8 and 10, and between gestation days 10 and 14. Thereafter, maternal food consumption was similar to controls.

Table 5.6.2/02-3: Mean maternal food consumption in rabbits administered BCS-CN88460 by oral gavage during gestation

		BCS-CN88460, dose in mg/kg bw/day			
Gestation day		**0**	**10**	**70**	**500**
6-8	g/day	149.3	168.1	160.3	127.2
	% control		112.6	107.4	85.2
8-10	g/day	162.7	170.2	159.7	126.4
	% control		104.6	98.1	77.7
10-14	g/day	143.7	150.6	141.0	123.8*
	% control		104.8	98.1	86.1
14-18	g/day	129.2	145.1	135.1	120.5
	% control		112.3	104.6	93.3
18-22	g/day	145.9	149.2	136.4	135.8
	% control		102.3	93.5	93.1
22-26	g/day	99.7	114.2	110.0	103.2
	% control		114.6	110.4	103.6
26-29	g/day	99.3	108.6	100.8	100.9
	% control		109.4	101.5	101.6

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

E. NECROPSY

There was no effect on liver weight at 10 or 70 mg/kg bw/day. At 500 mg/kg bw/day, mean liver weight was statistically significantly increased relative to controls. There were no treatment-related macroscopic findings in dams at scheduled necropsy.

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Table 5.6.2/02-4: Mean maternal carcass weight, liver weight, and thyroid weight after oral gavage administration of BCS-CN88460

		BCS-CN88460, dose in mg/kg bw/day			
		0	10	70	500
Maternal carcass weight	g	3306.3	3344.4	3287.0	3291.5
	% control		101.2	99.4	99.6
Liver wt	g	94.5	93.7	101.4	109.4**
	% control		99.2	107.3	115.8

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

F. CAESAREAN SECTION DATA

There were no treatment-related effects at any dose level on pregnancy rate, pre-implantation or post-implantation data, or on fetal data.

Table 5.6.2/02-5: Caesarean section data from dams administered BCS-CN88460 by oral gavage during gestation

	BCS-CN88460, mg/kg bw/day				
Maternal data	**0**	**25**	**125**	**625**	**HCD**
No. Animals assigned	23	23	23	23	NA
No. animals pregnant	23	22	23	23	NA
Pregnancy rate, %	100	96	100	100	NA
No. Animals non-pregnant	0	1	0	0	NA
Maternal wastage:					NA
Intercurrent death or sacrifice, total	0	0	0	2	NA
Intercurrent death or sacrifice, pregnant	0	0	0	2	NA
Premature delivery	0	0	0	0	NA
Intercurrent death or sacrifice, non-pregnant	0	0	0	0	NA
Abortions	0	0	0	2	NA
Uterine data at scheduled sacrifice:					NA
Total no. corpora lutea	279	248	276	240	NA
Corpora lutea per dam	12.1	11.3	12.0	11.4	10.67-12.81
Total no. implantations	243	199	226	204	NA
Implantations per dam	10.6	9.0	9.8	9.7	9.10-10.95
Total no. litters	23	22	23	21	NA
Total no. live fetuses	226	189	211	186	NA
Live fetuses per dam	9.8	8.6	9.2	8.9	8.23-9.90
Total no. dead fetuses	0	0	0	0	NA
Total no. early resorptions	10	6	8	6	NA
Early resorptions per dam	0.4	0.3	0.3	0.3	0.190-1.000
Total no. late resorptions	7	4	7	12	NA
Late resorptions per dam	0.3	0.2	0.3	0.6	0.000-0.318
Litters with total resorptions	0	0	0	0	NA
Mean fetal wt, g, combined sexes	39.7	41.8	39.9	39.7	35.05-41.37
Mean fetal wt, g, males	40.0	42.6	41.5	39.1	35.37-42.33
Mean fetal wt, g, females	39.5	41.1	37.7	39.9	34.57-40.85
Sex ratio, % males	45.6	47.1	49.3	44.1	NA
Sex ratio, % males per litter	45.1	45.8	51.0	44.4	40.3-56.6
Pre-implantation loss per dam, %	12.2	18.9	15.7	14.3	9.08-23.78
Post-implantation loss per dam, %	6.1	4.7	8.6	8.8	5.47-19.25

G. FETAL FINDINGS

There were no treatment-related malformations noted at fetal external, visceral, or skeletal observation.

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Table 5.6.2/02-6: Summary of fetal malformations

mg/kg bw/day	Dam	Fetus	External	Visceral	Skeletal
0	1F3155	7	Hindpaws (bi): malrotated: inward (severe)	No malformation	No malformation
	1F3159	9	No malformation	Aortic arch: dilated Left ventricle: dilated Right ventricle: small	No malformation
	1F3159	10	No malformation	Cleft palate (partial, communication with nasal cavity)	No malformation
	1F3164	1	No malformation	Ventricular septum defect in cranial region Cardiomegaly Ascending arch and aortic arch: dilated Fluid-filled abdomen	No malformation
	1F3165	4	No malformation	No malformation	1st to 5th sternebrae: fused 13th thoracic rib (bi): short (uni) Extra sternebral ossification site
10	2F3176	2	No malformation	Ventricular septum defect in median region Ascending aorta: narrowed Left ventricle: small	No malformation
	2F3182	7	No malformation	Renal pelvis (uni): dilated (severe) Ureter: retrocaval	No malformation
	2F3192	1	No malformation	No malformation	5th sternebra: unossified 13th thoracic rib (bi) 11th thoracic rib (uni): absent 11th thoracic vertebra: hemivertebra 10th thoracic centrum and 11th hemicentrum: fused 12th rib (uni): branched Insertion point (uni) of pelvic girdle on 2nd sacral vertebra 1st metacarpal: incomplete ossification

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Table 5.6.2/02-6 (cont.): Summary of fetal malformations

mg/kg bw/day	Dam	Fetus	External	Visceral	Skeletal
70	3F3196	6	No malformation	Cerebral lateral ventricle (bi): dilated (severe) Kidney (bi): fused and malpositioned (caudal position) Ureter (bi): short	No malformation
	3F3197	4	No malformation	Ascending aorta and aortic arch: dilated Pulmonary trunk: atresia Left ventricle: enlarged Right ventricle: small	No malformation
	3F3201	4	No malformation	No malformation	4th sternebra: incomplete ossification 5th sternebra: unossified 13th thoracic rib (bi). 9th thoracic arch (uni): small 9th and 10th ribs (uni): fused 9th and 10th thoracic centrum: fused
	3F3206	11	Snout: short (severe) Abdomen distended	No malformation	Parietal (uni): split Hyoid centrum: incomplete ossification Atlas centrum: unossified 4th cervical centrum: unossified 5th and 7th cervical centrum: incomplete ossification 1st sternebra: misshapen 6th sternebra: unossified 13th thoracic ribs (bi) 2nd and 3rd thoracic centrum: split 4th and 5th thoracic centrum: bipartite 2nd and 4th thoracic arch (uni): incomplete ossification Insertion point (bi) of pelvic girdle on 2nd sacral vertebra

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Table 5.6.2/02-6 (cont.): Summary of fetal malformations

mg/kg bw/day	Dam	Fetus	External	Visceral	Skeletal
500	4F3231	8	No malformation	Retina (uni): fold (severe) Vitreous chamber (uni): small Thymic remnant present (uni)	No malformation

Variations noted at external examination included outward malrotated forepaws, short tail, and distended abdomen.

Table 5.6.2/02-7: Incidence of external fetal variations

		BCS-CN88460, dose in mg/kg bw/day				
		0	**10**	**70**	**500**	**HCD, %**
N examined	Litters	23	22	23	21	
	Fetuses	226	189	211	186	
Abdomen distended	Litters	1	0	0	0	
	%	4.3	0.0	0.0	0.0	0.0-4.5
	Fetuses	1	0	0	0	
	%	0.4	0.0	0.0	0.0	0.0-0.5
Forepaws (uni/bi): malrotated: outward	Litters	0	1	1	2	
	%	0.0	4.5	4.3	9.5	0.0-13.0
	Fetuses	0	1	1	2	
	%	0.0	0.5	0.5	1.1	0.0-1.0
Tail: short	Litters	0	0	0	1	
	%	0.0	0.0	0.0	4.8	0.0-4.5
	Fetuses	0	0	0	1	
	%	0.0	0.0	0.0	0.5	0.0-0.5
Head: subcutaneous hemorrhage	Litters	0	0	0	1	
	%	0.0	0.0	0.0	4.8	none
	Fetuses	0	0	0	1	
	%	0.0	0.0	0.0	0.5	none

At visceral examination, a few findings were noted with an increased incidence in at least one of the treatment groups. However, these findings were within or marginally outside of the historical control data, did not show a relationship to treatment, and / or were not statistically significant. Although the incidence of retrocaval ureter is statistically significantly increased at 10 mg/kg bw/day, the absence of a dose relationship suggests that this finding is not related to treatment.

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Table 5.6.2/02-8: Incidence of fetal visceral variations

		BCS-CN88460, dose in mg/kg bw/day				
		0	**10**	**70**	**500**	**HCD, %**
N examined	Litters	23	22	23	21	
	Fetuses	226	189	211	186	
	Heads	108	90	101	89	
Retina (uni/bi): fold	Litters	1	5	2	3	
	%	4.35	22.7	8.7	14.3	0-14.3
	Fetuses	2	7	3	3	
	%	1.85	7.78	2.97	3.37	0-3.3
Caudate lung lobe: absent	Litters	1	2	2	2	
	%	4.35	9.09	8.7	9.52	0-30.0
	Fetuses	1	3	5	2	
	%	0.44	1.59	2.37	1.08	0-8.1
Ureter (uni): retrocaval	Litters	0	6	2	30	
	%	0.0	27.3**	8.7	14.3	0-19.0
	Fetuses	0	8	2	4	
	%	0.0	4.23**	0.95	2.15	0-3.2

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

There were no treatment-related findings noted at fetal skeletal examination. All of the skeletal variations observed occurred as isolated findings, or were observed with no dose-relationship, and / or were within the in-house historical control data range, and were thus considered to be incidental.

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Table 5.6.2/02-9: Incidence of fetal skeletal variations

		BCS-CN88460, dose in mg/kg bw/day				
		0	**25**	**125**	**625**	**HCD, %**
N examined	Litters	23	22	23	21	
	Fetuses	226	189	211	186	
	Heads	118	99	110	97	
Nasal (uni/bi) / frontal (uni) / parietal (uni/bi): split	Litters	2	4	3	3	
	%	8.7	18.2	13.0	14.3	0.0-47.6
	Fetuses	2	4	4	3	
	%	1.69	4.04	3.64	3.09	0.0-12.5
Anterior and / or posterior fontanelles: enlarged	Litters	1	1	3	2	
	%	4.35	4.55	13.0	9.52	0.0-20.0
	Fetuses	1	2	3	2	
	%	0.85	2.02	2.73	2.06	0.0-3.5
Hyoid centrum: incomplete ossification or unossified	Litters	6	8	12	6	
	%	26.1	36.4	52.2	28.6	4.8-60.0
	Fetuses	10	12	19	9	
	%	8.41	12.1	17.3*	9328	1.1-19.7
Extra ossification point (uni) and / or cervical rib (uni/bi): short on 7th cervical vertebra	Litters	3	4	1	4	
	%	13.0	18.2	4.35	19.0	4.2-23.8
	Fetuses	3	4	1	4	
	%	1.33	2.12	0.95	2.15	0.5-5.3
Extra sternebral ossification site	Litters	0	0	2	1	
	%	0.0	0.0	8.7	4.76	0.0-10.5
	Fetuses	0	0	2	3	
	%	0.0	0.0	0.95	1.61	0.0-1.2
5th and / or 6th sternebrae: unossified	Litters	10	13	11	12	
	%	43.5	59.1	47.8	57.1	22.7-76.2
	Fetuses	21	33	31	24	
	%	9.29	17.5*	14.7	12.9	4.2-31.5
1st metacarpal(s): incomplete ossification or unossified	Litters	12	5	6	11	
	%	52.2	22.7	26.1	52.4	9.5-60.9
	Fetuses	19	13	21	22	
	%	8.41	6.88	9.95	11.8	1.2-14.9

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Only the findings of "hyoid centrum incomplete ossification or unossified" and "5th or 6th sternebrae unossified" were statistically significant. However, in the absence of any dose relationship and as the statistically significant values were well within the historical control data, these observations are considered not to be treatment related.

H. DEFICIENCIES

No specific deficiencies exist in this study.

III. CONCLUSIONS

Based on maternal toxicity as evidenced by at least one abortion, statistically significant body weight loss, decreased food consumption, decreased mean maternal body weight change, and increased liver weight, the maternal NOAEL is set at 70 mg/kg bw/day. In the absence of any findings in fetuses which were related to treatment, the NOAEL for developmental toxicity is set at 500 mg/kg bw/day.

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CA 5.7 Neurotoxicity studies

Table 5.7-1: Summary of neurotoxicity studies with BCS-CN88460

Study	NOAEL	LOAEL	Effects
Acute neurotoxicity study Oral gavage dosing 0, 200, 600, 2000 mg/kg bw/day [redacted]; 2017; M-594177-01-1	Systemic NOAEL: 2000 mg/kg bw Neurotoxic NOAEL: 2000 mg/kg bw	> 2000 mg/kg bw/day	None

In the acute neurotoxicity study, BCS-CN88460 was administered by oral gavage at doses of 0, 200, 600, and 2000 mg/kg bw. Neurobehavioral assessments comprising a functional observation battery and motor activity measurements were carried out approximately one hour after dosing and again on study day 7 and 14. There were no mortalities or clinical signs, no were there any effects on various measures of neurotoxicity, on reflexes, on motor activity, or on either macro- or microscopic examination of the brain and nervous tissues. BCS-CN88460 was therefore not neurotoxic at the limit dose of 2000 mg/kg bw in the rat.
In the absence of any neurotoxic effect in the acute neurotoxicity study, a 90-day neurotoxicity study was not conducted. There is no structural relationship to any chemical class which causes delayed neurotoxicity, nor did repeat-dose studies show any indications of delayed neurotoxicity, and thus a specific study for delayed neurotoxicity of BCS-CN88460 was not conducted.

CA 5.7.1 Neurotoxicity studies in rodents

Report: KCA 5.7.1/01; [redacted]; 2017; M-594177-01-1
Title: BCS-CN88460 - An acute neurotoxicity study in the rat by oral administration
Report No.: SA 15004
Document No.: M-594177-01-1
Guideline(s): OECD guideline 424 (September, 1997); US EPA OCSPP guideline number 870.6200; MAFF in Japan Notification 12 Nousan No 8147 (November, 2000)
Guideline deviation(s): none
GLP/GEP: yes

Executive Summary
BCS-CN88460 was administered once by oral gavage to separate groups of Wistar rats (12 per sex per group) at dose levels of 0, 200, 600, and 2000 mg/kg bw. Neurotoxicity assessment including a functional observational battery (FOB) and spontaneous motor activity was performed on four occasions (during pre-study phase, approximately 6 hours after dosing, and then 7 and 14 days after dosing). All surviving animals were subjected to a complete necropsy. At least 6 animals per sex per group were subjected to neuropathological investigation with selected organs weighed and a range of organs fixed and examined microscopically.

Up to and including the highest dose tested of 2000 mg/kg bw, there was no mortality or any treatment-related daily clinical signs and there were no treatment-related effects on body weight or brain weight parameters, nor were there any effects at macroscopic examination. In addition, no treatment-related effects were observed in any of the neurotoxicology endpoints, including neuropathological examination in either sex.

In conclusion and based on these results, the dose level of 2000 mg/kg bw BCS-CN88460 was considered to the NOAEL in this study for both males and females.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% (w/w)
CAS #	1255734-28-1
Stability of test compound:	Until 11 June 2015

2. Vehicle and / or positive control: vehicle: 0.5% aqueous methylcellulose 400

3. Test animals:	
Species:	rat
Strain:	Wistar Rj:WI(IOPS HAN)
Age:	7-8 weeks of age
Weight at dosing:	295-361g for males; 179-254g for females
Source:	[redacted]
Acclimation period:	At least 20 days
Diet:	A04C-10 from [redacted], France
Water:	Filtered and softened tap water from the municipal water supply, ad libitum except during neurobehavioral testing
Housing:	Single housing in suspended polycarbonate cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15/hour
Photoperiod:	12 hours dark / 12 hours light

B. STUDY DESIGN AND METHODS

1. In life dates: 11 February 2015 – 20 March 2015

2. Dose preparation and analysis

The test item was suspended in aqueous 0.5% methylcellulose 400 to provide the required concentration. There was one preparation for each concentration for the study, and the formulations were stored at 5°C ± 3°C until use.

The stability of the test item in the vehicle has been demonstrated in a previous study at concentrations of 0.5 and 150 g/L for at least 27 days under similar conditions of usage and storage. In addition, the stability of the test item at 200 g/L was demonstrated in the present study for at least 28 days in similar conditions.

The homogeneity of the test item in the formulation was verified on the preparation at the lowest and highest concentrations, to demonstrate adequate formulation procedures. The concentration of the test item in the dosing formulation was verified for each dose level. The mean values obtained from the homogeneity check were taken as measured concentrations.

The homogeneity analyses showed results at 97-102% of the nominal concentration, while the concentration analyses showed that the measured concentrations were at 98-101% of

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nominal concentrations. As these were within the in-house target range of 90-110% of the nominal concentration, the formulations were considered to be acceptable for use.

3. Animal assignment and treatment

On the day of randomization, all animals were weighed. A computerized randomization procedure (Pristima, version 6.3.2 build 17, Xybion Corp.), which ensured a similar body weight distribution among groups for each sex, was used to select animals for the study from the middle of the weight range of the available animals. Selected animals were within ±20% of the mean body weight for each sex on the day of randomization.

In an acute oral toxicity study (M-485872-01-1, referenced in this Summary MCA Section 5 under Point 5.2.1/01), animals were dosed with BCS-CN88460 by oral gavage at a single dose level of 2000 mg/kg bw. No clinical signs or effects were noted during the study, including during the six hours following dosing. Based on these data, the doses selected for this acute neurotoxicity study were 0, 200, 600, and 2000 mg/kg bw.

Each animal was administered either the vehicle (0 mg/kg bw) or BCS-CN88460 suspended in 0.5% aqueous methylcellulose, formulated to the appropriate concentration. Dosing solutions were administered by oral gavage at a volume of 10 ml/kg bw, based on the most recent individual body weight.

Animals were checked for moribundity and mortality twice daily (once daily on weekends and public holidays), and all animals placed on study were observed for clinical signs at least once daily. The nature, onset, severity, duration, and recovery of clinical signs were recorded. Cages and cage trays were inspected daily for evidence of ill-health such as blood or loose feces.

During the pre-study phase and on study days 1, 7, and 14, a neurobehavioral assessment comprising a functional observation battery and motor activity measurement was conducted. In the absence of any clinical signs in the acute toxicity study at 2000 mg/kg bw, the neurobehavioral assessment on study day 1 was carried out approximately 1 hour after dosing. All animals were individually tested, with the order of animal testing randomly determined. The technicians involved in the conduct of this study were blinded with respect to the group assignment of each animal and were responsible for testing the same animals at each time point. The functional observation battery included:

- Home cage observation – while the animal was in its home cage, observations were collected regarding posture, piloerection, involuntary motor movements, gait abnormalities, vocalizations, or any abnormal behavior;
- Observation during handling – including ease 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 observation – each animal was individually observed in an open field for 2 minutes for piloerection, respiration, arousal, gait abnormalities, posture, involuntary motor movements, stereotypic movements, vocalizations, and number of rearings and urine or feces spots;
- Reflex and physiologic observations / measurements, including –
    - Pupil size,
    - Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary constriction after a narrow beam of light was focused on the eyes),
    - Surface righting reflex (by putting the animal on its back and evaluating its ability / rapidity to resume 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),

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- Flexor reflex (by pinching the toes and evaluating the presence / strength of the flexor response of each hindlimb),
- Auditory startle response (by evaluating the response to an auditory stimulus),
- Tail pinch response (by pinching the tail with a forceps and evaluating the animal reaction),
- Grip strength (measuring the fore- and hind-limb grip strength of animals quantitatively, using a grip strength apparatus equipped with one pull and one push strain gauge),
- Landing foot splay (by dropping the animal from approximately 30 cm above a padded surface, and marking, measuring, and recording the hindlimb foot splay),
- Body weight
- Rectal temperature.

The exploratory motor activity was measured individually for each animal using an automated photocell recording apparatus designed to quantify spontaneous exploratory activity in a novel environment. Exploratory motor activity was recorded during the first 60 minutes, with data collected at regular intervals throughout the session (for technical reasons, the activity was recorded for 65 minutes although only the first 60 minutes were analyzed).

Each animal was weighed on study day 1 prior to dosing, and then weekly during the study period as part of the FOB. Animals were also weighed prior to scheduled necropsy to collect terminal body weight.

For the animals used for neuropathology, on study days 15, 16, or 17, the first six animals at each dose level were selected for perfusion and tissue collection, with replacement of animals which were inadequately perfused. These animals were deeply anesthetized by inhalation of isoflurane and then euthanized by exsanguination during intravascular perfusion with a fixative solution. Prior to anesthesia, the animals were administered heparin sodium (60 mg/kg bw) by intraperitoneal injection. The perfusion via the left ventricle consisted of a flush with phosphate buffer, followed by the fixative solution (a solution of 4% formaldehyde and 1% glutaraldehyde in phosphate buffer). For these animals, necropsy examinations were limited to external and neuropathological observations.

In perfused animals, the entire carcass of the animal was post-fixed after preparation, with brain and the cervical and lumbar regions of the spinal cord exposed by removal of bone after perfusion fixation. The brain of perfused animals was weighed after post-fixation. The brain, dorsal root ganglia and spinal nerve roots from cervical and lumbar swellings, gasserian ganglia, gastrocnemius muscle, peripheral (sciatic, tibial, and sural) nerves, spinal cord (cervical, thoracic, and lumbar), and any macroscopic lesions in neural tissue or skeletal muscle were post-fixed in the solution used for the perfusion. The eyes and optic nerves were fixed in Davidson's fixative.

The remaining, non-perfused animals were deeply anesthetized by isoflurane inhalation, euthanized by exsanguination, and necropsied. The necropsy included the macroscopic examination of the external surfaces, all orifices, and all major body cavities. Significant macroscopic lesions were recorded but not sampled. No organs were collected from these animals.

Organs and tissues collected from animals in this study were processed for histopathology and embedded in paraffin wax or glycol methacrylate as appropriate. Histological slides were prepared for tissues from control and high-dose male and female rats.

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The brain was trimmed in a standard manner using a metal rodent brain matrix. The resulting eight coronal sections were representative of the following major brain regions: olfactory bulbs, cerebral cortex, caudate putamen, hippocampus, thalamus, hypothalamus, midbrain, and cerebellum, pons, and medulla oblongata.

The brain, spinal cord, eyes, optic nerves, and gastrocnemius muscle were embedded in paraffin wax, sectioned at approximately 4 µm, and stained with hematoxylin and eosin. Dorsal root ganglia (including dorsal and ventral root fibers) from the cervical and lumbar swellings, gasserian ganglia, and peripheral nerves (sciatic, tibial, and sural) were embedded in glycol methacrylate, sectioned at approximately 2-3 µm, and stained with Lee's stain.

All named organs from all animals in control and high-dose groups were examined using light microscopy. As no evidence of neuropathological effects was observed in the high dose males or females, no further analysis was performed.

4. Statistics

Means and standard deviations for body weight parameters, body weight change parameters, terminal body weight, absolute and relative organ weight parameters, exploratory motor activity, grip strength, landing foot splay, and rectal temperature were calculated for all groups. Statistical analyses were carried out separately for males and females, and group means were compared at the 5%, 1% and 0.01% levels of significance. Statistical analyses were carried out using Pristima version 6.3.2 build 17.

For body weight change parameters, terminal body weight, absolute and relative organ weight parameters, exploratory motor activity, grip strength, landing foot splay, and rectal temperature, the heterogeneity of variances between the groups was checked by Bartlett's test. Where no significant heterogeneity was detected, an analysis of variance was carried out. If the result was not significant, no further statistical analysis was conducted. Where the analysis of variance was significant, a 2-sided Dunnett test was used to determine the statistical significance of the result. If heterogeneity of variances was present, a Kruskal-Wallis test was used. If this was not significant, no further statistical analysis was conducted. If the Kruskal-Wallis test was positive, a 2-sided Dunn test was used to determine the statistical significance of the result.

For body weight parameters, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on log-transformed data. If the ANOVA on log-transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on log-transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

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II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities in either males or females at any dose throughout the study.

B. CLINICAL OBSERVATIONS

There were no treatment-related clinical signs observed at the daily observations throughout the study in any treated group compared to the controls.

C. FUNCTIONAL OBSERVATION BATTERY (FOB)

There were no treatment-related effects at any dose level in either males or females on home cage observations, observations during handling, open-field behavior, or sensory reactivity tests.

D. NEUROTOXICOLOGICAL ASSESSMENT

There were no relevant changes in grip strength, landing foot splay, rectal temperature, or body weight or body weight gain in any treated group compared to controls at any session. A few changes were noted, including:

- On day 7, a lower mean landing foot splay and a lower mean forelimb grip strength in males at 200 mg/kg bw;
- On day 14, a lower mean hindlimb grip strength in males at 2000 mg/kg bw;
- On day 14, a greater mean landing foot splay in females at 2000 mg/kg bw.

Table 5.7.1/01-1: Rectal temperature, landing foot splay, and fore- and hindlimb grip strength in male and female rats administered BCS-CN88460 by oral gavage

		BCS-CN88460, dose in mg/kg bw							
		Males				Females			
		0	200	600	2000	0	200	600	2000
Body wt, g	Pre-study	265.4	267.2	266.3	266.8	199.1	198.4	196.9	198.1
	1	323.8	324.5	322.8	319.0	222.9	217.0	223.8	224.9
	7	354.0	356.9	354.4	353.5	233.3	235.3	236.3	239.7
	14	378.3	391.1	391.1	383.2	247.5	250.8	248.3	250.5
Rectal temp., °C	Pre-study	38.05	38.03	38.07	37.94	38.35	38.39	38.31	38.36
	1	38.10	38.04	38.20	38.00	38.61	38.27	38.25	38.18
	7	38.05	38.07	38.03	38.05	38.88	38.55	38.73	38.81
	14	38.05	37.94	38.04	37.93	38.95	38.73	38.65	38.86
Landing foot splay, cm	Pre-study	9.05	7.12*	6.88*	7.29	6.56	7.62	7.29	7.30
	1	10.06	8.68	9.59	9.38	7.74	7.81	8.33	8.71
	7	10.59	8.46*	9.02	10.29	6.90	7.68	7.91	8.14
	14	10.37	8.88	9.11	8.91	7.54	7.98	8.72	9.34*
Forelimb grip strength, g	Pre-study	526.8	479.4	529.0	500.6	494.4	547.8	555.8	498.8
	1	667.1	592.3	592.2	573.7	561.4	553.0	625.5	598.7
	7	666.1	500.5**	555.0	590.8	481.9	525.4	545.3	527.1
	14	574.8	549.9	594.3	583.2	427.9	463.1	533.5	432.9
Hindlimb grip strength, g	Pre-study	416.5	421.3	398.8	378.1	430.3	451.3	490.8	438.3
	1	485.3	502.0	472.0	449.8	429.2	454.3	481.1	462.8
	7	534.8	514.3	483.8	469.6	395.4	447.3	449.9	430.3
	14	605.5	618.1	541.1	483.8**	412.8	414.7	449.4	409.4

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

However, it is clear that there was no consistency across either time period or dose group, nor was there a dose relationship for the measurement of landing foot splay and forelimb grip strength in males at 2000 mg/kg bw on day 7. In the absence of such a relationship to dose, of

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consistency across sexes, and repeatability across time periods, these observations are considered to be not relevant to treatment. This is supported by the occasional statistically significant increases or decreases in measurements made during the pre-study examinations.

No relevant changes were recorded in overall motor activity in any test groups compared to the control group at any time point. In addition, the general pattern of motor activity within each test session was similar between test groups and the control group, showing no indication of a treatment-related effect.

E. BODY WEIGHT

There were no effects on either body weight or body weight gain in either males or females at any dose level compared to controls. There was a slight tendency towards an increase in body weight gain among treated groups; however, this is attributed to a high heterogeneity of individual values, especially in control groups, leading to lower mean values in controls. Additionally, there was no statistical significance of the increase and no relationship to dose, thus the slight changes observed in body weight were considered to be not relevant and not related to dose.

Table 5.7.1/01-2: Mean body weight and body weight change in males and females administered a single dose of BCS-CN88460

		BCS-CN88460, dose in mg/kg bw							
		Males				Females			
		0	200	600	2000	0	200	600	2000
Body wt, g	1	325	326	323	319	224	218	224	225
	7	354	357	354	354	233	235	236	240
	14	378	391	391	383	248	254	248	251
Body wt gain, g	1-7	29	31	31	35	10	18	13	14
	1-14	53	65	68	64	24	33	25	25

F. FOOD CONSUMPTION

Food consumption was not measured in this study.

G. NECROPSY

There was no effect on mean terminal body weight or mean brain weight in treated males or females at any dose level when compared to controls.

No macroscopic findings were observed in any dose group in either males or females, either in animals of the neuropathology group or in other animals.

No treatment-related microscopic findings were noted in either males or females at any dose level.

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Table 5.7.1/01-3: Mean terminal body weight, absolute and relative brain weight, and macro- and microscopic observations in the nervous tissues examined in male and female rats administered BCS-CN88460 by oral gavage

	BCS-CN88460, dose in mg/kg bw							
	Males				Females			
	0	200	600	2000	0	200	600	2000
N examined	6	6	6	6	6	6	6	6
Terminal body wt, g	380.55	391.62	397.65	387.18	249.93	256.12	249.05	256.82
Brain wt, g	1.939	1.925	1.942	1.964	1.815	1.838	1.789	1.856
Brain wt, % body wt	0.512	0.492	0.488	0.509	0.728	0.718	0.725	0.724
Macroscopic examination								
N examined	3	3	3	4	5	4	3	4
Brain								
Focus(i), red	0	0	0	0	0	0	1	0
Microscopic examination								
N examined	6	0	0	6	6	0	0	6
Brain, level 3								
Hydrocephalus	0			1	0			0
Brain, level 4								
Hydrocephalus	0			1	0			0
Mononuclear cell infiltrate, focal	0			0	0			1
Brain, level 5								
Hydrocephalus	0			1	0			0
Brain, level 6								
Hydrocephalus	0			1	0			0
Mononuclear cell infiltrate: choroid plexus	0			0	1			0
Dorsal root ganglion								
Mononuclear cell infiltrate, focal	1			0	0			0
Sciatic nerve, left								
Axonal degeneration, focal	1			2	2			2
Sciatic nerve, right								
N examined	0			0	1			0
Axonal degeneration, focal	-			-	1			-
Spinal cord, cervical								
Mononuclear cell infiltrate, focal	0			0	1			0
Spinal nerve roots								
Axonal degeneration, focal	0			0	1			0
Sural nerve, left								
Axonal degeneration, focal	2			0	0			0
Tibial nerve, left								
Axonal degeneration, focal	1			1	1			1

H. DEFICIENCIES

Food consumption was not measured in this study. However, in the absence of any indication of a neurotoxic effect on behavior or on reflexes, and with no changes in body weight or body weight gain in either males or females, it is not likely that this omission affected the validity or the conclusions of the study.

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III. CONCLUSIONS

Up to and including the highest dose tested, 2000 mg/kg bw, there was no mortality nor were there any clinical signs, and there were no treatment-related effects on body weight or brain weight parameters. Additionally, no treatment-related effects were observed in any of the neurotoxicology endpoints in either males or females.

Based on these results, the dose level of 2000 mg/kg bw of BCS-CN88460 was considered to be the NOAEL in this study in both males and females.

CA 5.7.2 Delayed polyneuropathy studies

BCS-CN88460 is not a member of a chemical class which causes delayed neuropathology, nor were any neuropathological findings observed in any repeat-dose study. Thus, delayed neuropathology studies are not required.

CA 5.8 Other toxicological studies

CA 5.8.1 Toxicity studies of metabolites

Report: KCA 6.7.1/01, R.; [redacted], D.; [redacted], E.; 2018; M-612432-01-1
Title: Isoflucypram (BCS-CN88460): Evaluation of dietary metabolites and residue definition proposals
Report No.: M-612432-01-1
Document No.: M-612432-01-1
Guideline(s): none
Guideline deviation(s): –
GLP/GEP: **no**

This document summarizes the metabolites which have been conclusively identified and considered for inclusion in the risk assessment residue definition, estimates consumer exposure to each of those metabolites based on the field studies from representative uses, the field rotational crops study, and the livestock feeding studies, and evaluates the genotoxicity of the conclusively-identified metabolites via in silico methods.

The full list of metabolites (M01 through M76) and the matrices in which each of them is found is given in Document N3 (M-613310-01-1). This includes the rat metabolism studies, the livestock feeding studies, field studies, and field rotational crop studies, and lists all metabolites which were conclusively identified.

Of the metabolites listed in Document N3, those which were detected

- in food of plant origin,
  - ≥ 10% of the TRR and ≥ 0.01 mg/kg, or
  - <10% of the TRR but ≥ 0.05 mg/kg,

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- in plant feed items, at ≥ 10% of the TRR and ≥ 0.01 mg/kg, or
- in food of animal origin, at ≥ 10% of the TRR

were considered for potential inclusion into the residue definition for risk assessment. The list of these metabolites is shown in Table 1 of the above-referenced document M-612432-01-1.

Based on their occurrence in metabolism studies, a total of six metabolites (and their conjugates, which were summed with their respective aglyca) were selected for potential inclusion in the residue definition. These metabolites were BCS-CN88460-propanol (M01) and its conjugates, BCS-CN88460-2-propanol (M02) and its conjugates, BCS-CN88460-desmethyl-propanol (M06) and its conjugates, BCS-CN88460-desmethyl-1,2-propandiol (M07) and its conjugate, BCS-CN88460-desmethyl-carboxylic acid (M11), and BCS-CN88460-carboxylic acid (M12).

The estimated consumer exposure to each of the metabolites and its conjugates, in µg/kg bw/day and as a percentage of the acute and chronic TTC thresholds, is shown in Table 5.8.1-1 below:

Table 5.8.1-1. Estimated consumer exposure to metabolites of BCS-CN88460, via all matrices, in µg/kg bw/day and relative to the TTC thresholds for chronic and acute scenarios

	Chronic exposure		Acute exposure	
Metabolite	**µg/kg bw/day**	**% TTC threshold**	**µg/kg bw/day**	**% TTC threshold**
M01 and conjugates	0.019	1.3	0.24	4.7
M02 and conjugates	0.013	0.85	0.084	1.7
M06 and conjugate	0.009	0.61	0.31	0.63
M07 and conjugate	0.0003	0.02	0.007	0.14
M11	0.0003	0.02	0.011	0.21
M12	0.0160	1.1	0.061	1.2
Total	0.0582	3.9	0.713	8.58

The incidence of each of the aglyca listed above in the rat metabolism studies, and their coverage by the toxicology studies conducted with the parent, is detailed in Table 2 of the above-mentioned document M-612432-01-1. Because of the high structural similarity of these metabolites to the parent BCS-CN88460, as well as the detection of, for example M01, glucuronidated forms of M06, and downstream metabolites of M12 in the rat ADME studies, and the measurement of M11 in key studies conducted in the rat, mouse, dog, and rabbit with BCS-CN88460, it is considered that these metabolites are covered by toxicity studies conducted with the parent compound.

In order to evaluate the potential for genotoxicity of the metabolites and their conjugates, the structures of BCS-CN88460, the metabolites listed above, and their conjugates were assessed by in silico methods (Derek Nexus, Leadscope, Toxtree, TopKat, and Vega). The in silico predictions for the metabolites do not differ from those for the parent in models which have been shown to have a high reliability for agrochemical substances.

Genotoxicity studies with the metabolites are not necessary for the following reasons:

- BCS-CN88460 is neither mutagenic nor clastogenic.
- The metabolites are predicted to have the same genotoxicity profile as parent based on the in silico analyses.
- Consumer exposure is well below the chronic and acute TTC thresholds when considering:
  - Each aglycone and its conjugates, or
  - All metabolites and their conjugates considered together.

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Toxicological studies on the impurity BCS-CN45153

The impurity BCS-CN45153 was a development candidate which preceded BCS-CN88460 in the development process. The development of BCS-CN45153 was stopped at an early stage due to the adverse toxicological findings described below. Because of these adverse findings, BCS-CN45153 is to be considered a relevant impurity.

Table 5.8.1-2: Studies conducted on the impurity BCS-CN45153

Study	NOAEL	LOAEL	Effects at LOAEL
28-day rat study Dietary study 0, 200, 600, 1800 ppm [redacted]; 2011; M-416617-01-1	600 ppm 50 / 54 mg/kg bw/day males / females	1800 ppm 152 /145 mg/kg bw/day males / females	Decreased body weight / body weight gain, increased liver weight, hyaline droplet accumulation in males; uterine squamous metaplasia
28-day mouse study Dietary study 0, 400, 2000, 4500 ppm [redacted]; 2010; M-395125-01-1	400 ppm 64 / 73 mg/kg bw/day males / females	2000 ppm 337 /377 mg/kg bw/day males / females	Increased liver weight, centrilobular hepatocellular hypertrophy
Uterotrophic / vaginal opening assay Oral gavage administration [redacted]; 2011; M-403872-01-1	150 mg/kg bw/day	450 mg/kg bw/day	Advancement of vaginal opening

The toxicity of the impurity BCS-CN45153 was investigated in a 28-day rat study, a 28-day mouse study, and an uterotrophic assay in the immature female rat.

In the 28-day rat study, BCS-CN45153 was administered via the diet to male and female rats at concentrations of 0, 200, 600 and 1800 ppm (providing systemic doses of 0, 17.3, 50.0, and 152 mg/kg bw/day in males and 0, 17.6, 54.0, and 145 mg/kg bw/day in females). There were no effects at 200 or 600 ppm. At 1800 ppm, mean body weight and body weight gain were slightly reduced in both sexes. Liver weights were increased on both sexes, but there was no concurrent histopathological effect nor was there a biologically meaningful induction of either total cytochrome P450 or any of the enzyme activities measured. In the males, kidney weight was increased and accompanied by an increase in hyaline droplets and signs of nephropathy. A treatment-related increase in thyroid follicular cell hypertrophy was observed in males only. In females, uterine squamous metaplasia was noted in 2/5 animals, but there was no effect on the estrous cycle. The NOEL of this study was established at 600 ppm (50.0 mg/kg bw/day in males and 54.0 mg/kg bw/day in females).

A 28-day dietary toxicity study with BCS-CN45153 was conducted in the mouse, at concentrations of 0, 400, 2000, and 4500 ppm (providing systemic doses of 0, 64, 337, and 779 mg/kg bw/day in males and 0, 73, 377, and 819 mg/kg bw/day in females). No toxicologically relevant findings were noted at 400 ppm. At 2000 ppm, the only relevant observations were increased liver weight in both males and females, accompanied by an increase in the incidence of hepatocellular hypertrophy. At 4500 ppm, body weight and body weight gain were slightly reduced. The liver was the primary target organ of BCS-CN45153 in the mouse, with an increase in total cholesterol in females, decreased bilirubin concentration in males, and slightly increased alkaline phosphatase concentrations in males. Absolute

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and relative liver weights were increased in both sexes, accompanied by enlarged liver, increased hepatocellular hypertrophy, and increased single cell necrosis. The dietary concentration of 400 ppm (64 mg/kg bw/day in males, 73 mg/kg bw/day in females) was an NOAEL in males and NOEL in females.

In the immature rat uterotrophic assay, the compound was administered by oral gavage at doses of either 150 or 450 mg/kg bw/day for 20 days. Increased salivation was seen in both treatment groups, while at 450 mg/kg bw/day there was also a decrease in motor activity and lack of grooming. At 450 mg/kg bw/day, there was a clear reduction in age at first vaginal opening, and mean body weight at vaginal opening was decreased compared to control animals. There was no relevant effect on uterus weights at either dose. Based on these results, BCS-CN45153 interferes with pubertal development in the immature female rat.

Report:	KCA 5.8.1/01; [redacted]; 2011; M-416617-01-1
Title:	BCS-CN45153 - Exploratory 28-day toxicity study in the rat by dietary administration (summary report)
Report No.:	SA 10158
Document No.:	M-416617-01-1
Guideline(s):	not specified
Guideline deviation(s):	not specified
GLP/GEP:	**no**

Executive Summary

Shortly after the in-life phase of this study (15 September 2010-7 January 2011), the development of BCS-CN45153 was stopped and only a letter report of the 28-day rat study was produced. The entire text of the report is provided here.

BCS-CN45153 (batch number: NH18224-10-2 a beige powder, 98.4% w/w purity), a fungicide of the cypropamide family, was administered continuously via the diet to groups of Wistar rats (5/sex/group) for at least 28 days at concentrations of 200, 600 and 1800 ppm, corresponding to 17.3, 50.0 and 152 mg/kg/day in males and 17.6, 54.0 and 145 mg/kg/day in females, respectively. A similarly constituted group received untreated diet and acted as a control. All animals were observed for mortality and clinical signs daily, body weight and food consumption were measured weekly. A detailed physical examination was performed weekly throughout the study. Before necropsy a blood sample was collected from the retro-orbital venous plexus of each animal for hematology and clinical chemistry determinations. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically. The remaining portions of the liver were homogenized for microsomal preparations in order to determine cytochrome P-450 isoenzyme profile (EROD: ethoxyresorufin, PROD: pentoxyresorufin, BROD: benzoxyresorufin O-deethylase, LAH: lauric acid hydroxylase).

There were no mortalities, no treatment-related clinical signs, and no change in hematology parameters observed during the study.

At 1800 ppm:

In males, there were no effects on mean body weight throughout the study, except on the first week where a slight decrease of 6% ($p \leq 0.05$) was observed, corresponding to a reduced mean body weight gain/day of 26% ($p \leq 0.01$) and mean absolute body weight gain of 25% ($p \leq 0.01$), compared to the respective concurrent controls. Food consumption was not affected by the treatment, though food spillage was noted for few animals. At necropsy, mean absolute and relative liver weights were statistically significantly higher when compared to the controls (+21 to 22%, $p \leq 0.01$). These changes were not considered to be treatment-related since no relevant histopathological findings were observed (only 1 animal with minimal centrilobular hypertrophy) and as they were not correlated with an induction of the xenobiotic metabolizing enzymes. Mean absolute and relative kidney weights were

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statistically significantly higher when compared to the controls (+19%, p≤0.01). These changes were associated with relevant microscopic findings (higher incidence and severity of hyaline droplets with signs of nephropathy in 2/5 animals) and were thus considered to be treatment-related. In the thyroid gland, minimal follicular cell hypertrophy was observed in 3/5 males and was considered to be treatment-related. Liver enzyme activities were very slightly increased compared to the controls: PROD (2 fold), BROD (3 fold) and Cytochrome P450 level (1.3 fold).

In females, mean body weight was reduced throughout the 4 weeks of treatment from 12% to 7% compared to the controls (not statistically significant). This effect was essentially attributable to a mean absolute body weight loss of 1 g (p≤0.01) during the first week of the study compared to a gain of 22 g for the controls. Food consumption was decreased by 35% on week 1 (p≤0.01, spillage noted for 3/5 animals), 13% on week 2 (not statistically significant, spillage noted for 3/5 animals) and 27% on week 3 (not statistically significant, spillage noted for 3/5 animals). At necropsy, mean terminal body weight was 12% lower than the controls (not statistically significant). Mean liver to body weight ratio was statistically significantly higher when compared to the controls. In the absence of relevant microscopic findings, this change was considered to be incidental and not treatment-related. Lower mean total bilirubin concentrations were observed (-75%, p≤0.01) compared to the controls, but this change was considered not to be an adverse effect as it does not represent any functional impairment in the test organism. In the uterus, minimal squamous metaplasia in endometrial glands was noted in 2/5 females but the estrous cycle was not modified when compared to the controls. Liver enzyme activities were very slightly increased compared to the controls: BROD (4 fold) and Cytochrome P450 level (1.3 fold).

At 600 ppm:
There were no treatment-related changes.

At 200 ppm:
There were no treatment-related changes.

In conclusion, the dose level of 600 ppm (equating to approximately 50.0 and 54.0 mg/kg/day in males and females, respectively) was considered to be a No Observed Effect Level (N.O.E.L.) in both sexes.

Report:	KCA 5.8.1/02; [redacted]; 2010; M-395125-01-1
Title:	BCS-CN45153 - Preliminary 28-day toxicity study in the mouse by dietary administration
Report No.:	SA 09400
Document No.:	M-395125-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Executive Summary

BCS-CN45153 was administered continuously via the diet to groups of C57BL/6J mice (5/sex/group) for at least 28 days at concentrations of 400, 2000 and 4500 ppm (equating approximately to 64, 337 and 779 mg/kg body weight/day in males and 73, 377 and 819 mg/kg body weight/day in females). A similarly constituted group received untreated diet and acted as a control. Animals were observed daily for mortality and clinical signs. A detailed physical examination was performed at least weekly. Body weight and food consumption were recorded approximately weekly. Selected clinical chemistry parameters were determined at the end of the study. All animals were subjected to necropsy, selected organs weighed and a range of tissues were fixed and examined microscopically.

Dietary administration of BCS-CN45153 for at least 28 days to male or female C57BL/6J mice at dose level of 400, 2000 and 4000 ppm induced no mortality, nor clinical signs.

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At 4500 ppm
Mean body weight in males was marginally reduced by between 3 and 4% from study days 15 to 29, when compared to controls. In females, mean body weight was reduced by 4 to 8% throughout the study, when compared to controls. The effect was statistically significant on study day 15 only.

Between study days 8 and 15, there was a mean body weight gain of 0.08 g/day in males compared to 0.16 g/day in the controls and a mean body weight loss of 0.04 g/day in females compared to a gain of 0.08 g/day in the controls. Thereafter, body weight evolution was unaffected by treatment in either sex.

Mean absolute body weight gain was reduced by 36 to 76% throughout the study in females only, the effect being statistically significant between study days 8 and 15.

Mean food consumption was unaffected by treatment in males and was reduced in females by 16% between study days 8 to 15 and by 14% to 15% between study days 15 to 22 and 22 to 29, respectively.

Clinical chemistry assessment revealed slightly higher mean total cholesterol concentrations in females (+24%). In addition at this dose level, lower mean total bilirubin concentrations were observed in males (-70%), when compared to controls, but this change was considered not to be adverse effect as it does not represent any functional impairment in the test organism. Tendencies towards higher alkaline phosphatase activities were also seen in males and lower creatinine, total protein and albumin concentrations were observed in females, but these variations were considered not to be biologically or toxicologically relevant as the individual values were within the normal range for mouse of this strain and age.

Mean absolute and relative liver weights were significantly increased by 37 to 44% in males and by 32 to 40% in females when compared to controls. At necropsy, enlarged liver was observed in 3/5 males and 1/5 females. At microscopic evaluation, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was noted in 5/5 males and 4/5 females. Minimal to slight hepatocellular single cell necrosis was found in 4/5 males and 3/5 females.

At 2000 ppm
Clinical chemistry assessment revealed lower mean total bilirubin concentrations (-60%) in males, when compared to controls, but this change was considered not to be adverse effect as it does not represent any functional impairment in the test organism. In addition, tendencies towards lower creatinine, and total protein concentrations were observed in females, but these variations were considered not to be biologically or toxicologically relevant as the individual values were within the normal range for mouse of this strain and age.

Mean absolute and relative liver weights were significantly increased by 19 to 23% in males and by 15 to 19% in females when compared to controls. At microscopic evaluation, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was noted in 3/5 males and 2/5 females.

At 400 ppm
Clinical chemistry assessment revealed lower mean total bilirubin concentrations in males, when compared to controls.

In conclusion, a dose level of 400 ppm of BCS-CN45153 following continuous dietary administration to C57BL/6J mice for at least 28 days was a No Observed Adverse Effect Level (NOAEL) in males (equating approximately to 64 mg/kg body weight/day) and a No Observed Effect Level (NOEL) in females (equating approximately to 73 mg/kg body weight/day).

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Light beige solid
Lot / Batch #:	NLL 8224-6-4
Purity:	97.6% (w/w)
CAS #	Not assigned
Stability of test compound:	Until 18 August 2011

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	mouse
Strain:	C57BL/6J
Age:	Approximately six weeks of age
Weight at dosing:	19.0-23.4g for males; 16.1-18.3g for females
Source:	[redacted]
Acclimation period:	6 days
Diet:	A04CPI-10, [redacted], France, ad libitum
Water:	Filtered and softened tap water from the municipal water supply ad libitum
Housing:	Individual housing in suspended stainless steel wire mesh cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15/hour
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN AND METHODS

1. In life dates: 31 March 2010-5 May 2010

2. Animal assignment and treatment

During the acclimatization phase, all animals were weighed at least weekly and subjected to a detailed physical examination once. On the day of assignment to groups, all animals were weighed. An automatic randomization procedure (XMS Path/Tox Version 4.2.2) was used to select animals for the study from the middle of the weight range of the available animals, ensuring a similar body weight distribution among groups for each sex (± 20% of the mean body weight on the day of randomization). Animals were assigned permanent identification numbers within groups following randomization.

Groups of 5 male and 5 female mice were given the vehicle control diet or the appropriate diet mixture at concentrations of 0, 400, 2000, and 4500 ppm. These dose levels were selected after evaluation of a 14-day dietary study in which BCS-CN45153 was administered via the diet to groups of 5 male and 5 female mice at concentrations of up to 6000 ppm.

3. Diet preparation and analysis

The test item was incorporated into the diet by dry mixing to provide the required dietary concentrations. There was one preparation for each concentration. When not in use, the diet formulations were stored frozen.

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The concentration of the test item in the diet was verified for each concentration at preparation. The stability of the test substance at relevant concentrations had been determined in a separate study (M-393802-01-1 referenced under KCA 4.1.2 and summarized in MCA Summary Section 4 under Point 4.2.1 (c)).

4. Statistics

Mean and standard deviation were calculated for each group, and all statistical analyses were carried out separately for males and females.

For body weight gain, terminal body weight, absolute and relative organ weights, and clinical chemistry parameters, mean and standard deviation were calculated for each group, and per time period for body weight gain / day parameters. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the man of the control group using the two-sided Dunn test.

For body weight and average food consumption per day, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on log-transformed data. If the ANOVA on log-transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on log-transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the man of the control group using the two-sided Dunn test.

C. METHODS

1. Observations

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

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2. **Body weight**
Each animal was weighed at least weekly during the acclimatization period, on the first day of test item administration, and then at weekly intervals throughout the treatment period. At scheduled necropsy, terminal body weight was collected by weighing animals which had been diet-fasted overnight.

3. **Food consumption and compound intake**
The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals in all groups during the study. Any food spillage was noted. The weekly mean achieved dosage intake in mg/kg bw/day for each week and for weeks 1-4 was calculated for each sex.

4. **Ophthalmoscopic examination**
Ophthalmoscopic examination was not conducted.

5. **Clinical chemistry**
On study day 30, 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 were anesthetized by inhalation of isoflurane. Blood was collected on clot activator for serum clinical chemistry determinations.

6. **Urinalysis**
Urinalysis was not conducted.

7. **Sacrifice and pathology**
On study day 30, all animals from all groups were anesthetized by inhalation of isoflurane and then exsanguinated. An approximately equal number of animals randomly distributed among all groups were sacrificed on each day. Animals were diet fasted overnight prior to sacrifice. All animals were necropsied, including the examination of all major organs, tissues, and body cavities. Microscopic abnormalities were recorded, sample, and examined microscopically.

At final sacrifice, weights were taken (paired organs were weighed together) of adrenal gland, brain, kidney, liver, ovary, spleen, testis, and uterus including cervix. The adrenal gland, epididymis, kidney, liver, ovary, pituitary gland, spleen, testis, thyroid gland with parathyroid gland, uterus including cervix, vagina, and any macroscopic findings were sampled and fixed by immersion in neutral buffered 10% formalin. Testis and epididymis were fixed in Davidson's fixative. Histological sections of these organs were prepared for all animals in all groups and stained with hematoxylin and eosin.

Histopathological examinations of all sampled organs (except parathyroid gland) were performed on all slides from all animals from control and high dose groups. Kidney, liver, thyroid gland, and macroscopic findings of all animals were examined in the intermediate dose groups.

II. RESULTS AND DISCUSSION

A. CLINICAL SIGNS AND MORTALITY

1. **Clinical signs of toxicity**
There were no treatment-related clinical signs observed during the study at any dose level in either sex.

2. **Mortality**
There were no mortalities during the study.

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B. BODY WEIGHT AND BODY WEIGHT GAIN

At 4500 ppm, mean body weight in males was marginally reduced during the second half of the study. In females, body weight was statistically significantly reduced throughout the study. Body weight gain was statistically significantly reduced at 4500 ppm between study days 8 and 15 in both males and females, and at the end of the study overall body weight gain was reduced in females compared to controls.

Table 5.8.1/02-1: Mean body weight and body weight gain in male and female rats administered BCS-CN45153 via the diet for four weeks

		BCS-CN45153, dietary concentration in ppm							
		Males				Females			
	Day	0	400	2000	4500	0	400	2000	4500
Body wt, g	1	21.5	20.6	20.7	20.8	17.4	17.5	17.2	17.1
	8	21.8	21.4	21.9	21.3	18.5	18.4	18.6	17.8
	15	23.0	22.4	22.3	21.9	19.1	19.0	18.9	17.5*
	22	23.5	22.6	22.8	22.7	19.6	19.7	19.2	18.4
	29	24.1	23.3	23.3	23.2	20.2	19.9	19.8	18.9
Body wt gain, g	1-29	2.6	2.7	2.6	2.4	2.9	2.8	2.6	1.8

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

C. FOOD CONSUMPTION AND COMPOUND INTAKE

In females at 4500 ppm, there was a slight reduction in food consumption from study day 8. There were no other effects on food consumption.

Table 5.8.1/02-2: Mean achieved dietary intake, in mg/kg bw/day of BCS-CN45153 in a mouse 4-week dietary study

	BCS-CN45153, dietary concentration in ppm		
	400	2000	4500
Males	64	337	779
Females	77	371	819

D. OPHTHALMOSCOPIC EXAMINATION

Ophthalmoscopic examination was not conducted in this study.

E. HEMATOLOGY, CLINICAL CHEMISTRY, AND URINALYSIS

1. Hematology

Hematological analysis was not conducted in this study.

2. Clinical Chemistry

The only changes in clinical chemistry parameters which were considered to be related to treatment were decreases in total bilirubin concentrations in males and total cholesterol in females.

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Table 5.8.1/02-3: Selected clinical chemistry parameters in male and female rats administered BCS-CN45153 via the diet for four weeks

	BCS-CN45153, dietary concentration in ppm							
	Males				Females			
Parameter	**0**	**400**	**2000**	**4500**	**0**	**400**	**2000**	**4500**
Bilirubin µmol/L	1.0	0.6	0.4*	0.3**	1.1	0.6	0.9	0.7
Cholesterol mmol/L	1.88	1.81	1.60*	2.51	1.50	1.45	1.29	1.86*

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

3. Urinalysis

Urinalysis was not conducted in this study.

F. SACRIFICE AND PATHOLOGY

1. Terminal body weights and organ weight

Terminal body weight was unaffected by dietary administration of BCS-CN45153 in either males or females. Absolute and or relative liver weights were statistically significantly increased from 2000 ppm in both males and females.

Table 5.8.1/02-4: Terminal body weight and absolute and relative liver weights in male and female mice administered BCS-CN45153 via the diet for four weeks

	BCS-CN45153, dietary concentration in ppm			
	Males			
	0	**400**	**2000**	**4500**
Terminal body wt, g	19.9	19.5	19.3	19.1
Liver wt, g	0.84	0.91	1.00*	1.15**
Liver to body wt, %	4.232	4.565	5.211*	6.025**
Liver to brain wt, %	190.618	208.113	234.892**	274.013**
	Females			
	0	**400**	**2000**	**4500**
Terminal body wt, g	16.3	16.1	16.1	15.8
Liver wt, g	0.72	0.70	0.83	0.95**
Liver to body wt, %	4.382	4.381	5.141*	6.041**
Liver to brain wt, %	160.344	161.436	190.850	224.928**

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$ *** $p \leq 0.001$

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2. Gross and microscopic pathology

At 4500 ppm, dark liver was noted in 3 of 5 males and one of five females.

Table 5.8.1/02-5: Incidence of macroscopic changes in the liver in male and female mice administered BCS-CN45153 via the diet for four weeks

	BCS-CN45153, dietary concentration in ppm			
	Males			
	0	**400**	**2000**	**4500**
Terminal body wt, g	19.9	19.5	19.3	19.1
Liver wt, g	0.84	0.91	1.00*	1.15**
Liver dark	0	0	0	3
	Females			
	0	**400**	**2000**	**4500**
Terminal body wt, g	16.3	16.1	16.1	15.8
Liver wt, g	0.72	0.70	0.83	0.95**
Liver dark	0	0	0	1

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$ *** $p \leq 0.001$

At microscopic examination the incidence of centrilobular hepatocellular hypertrophy and focal hepatocellular single cell necrosis were noted in both males and females from 2000 ppm.

Table 5.8.1/02-6: Incidence of microscopic changes in the liver of male and female mice administered BCS-CN45153 via the diet for four weeks

	BCS-CN45153, dietary concentration in ppm							
	Males				Females			
Parameter	**0**	**400**	**2000**	**4500**	**0**	**400**	**2000**	**4500**
Liver, N examined	5	5	5	5	5	5	5	5
Hepatocellular hypertrophy	0	0	3	5	0	0	2	4
Hepatocellular single cell necrosis	1	0	1	4	1	0	0	3

G. DEFICIENCIES

No specific deficiencies were noted in the study.

III. CONCLUSIONS

Effects of BCS-CN45153 after subacute dietary administration to male and female mice were limited to the liver. Observations which were increased in either a statistically or biologically significant manner were increased liver weight and in males only decreased bilirubin, and in both sexes an increase in the incidence of hepatocellular hypertrophy and hepatocellular single cell necrosis.

Based on these observations in the liver, the dose level of 400 ppm (approximately 64 mg/kg bw/day in males and approximately 73 mg/kg bw/day in females) was an NOAEL in males and an NOEL in males.

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Report: KCA 5.8.1/03; [redacted]; 2011; M-403872-01-1
Title: BCS-CN45153 - Evaluation in the immature rat uterotrophic assay coupled with vaginal opening
Report No.: SA 10312
Document No.: M-403872-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: **no**

Executive Summary
Groups of 6 female Sprague-Dawley rats, 19 days old, were dosed for at least 20 days with either the vehicle (group 1) or BCS-CN45153 (groups 2 and 3) at 150 or 450 mg/kg/day. Vaginal opening was recorded on Day 10 and daily thereafter. The uterine weight (wet and blotted) was recorded 24 hours after the end of the dosing period.

At 450 mg/kg/day, treatment-related clinical signs consisted of increased salivation, reduced motor activity and lack of grooming. There was no effect on the mean body weight. The mean age of first vaginal opening in animals was significantly reduced (≤ 30.3 days) compared to control animals (33.5 days). In addition, the mean body weight at vaginal opening was reduced by 20% compared to control animals (≤ 87.9 g v.s. 110 g). No significant effect was observed on uterus weights at necropsy.

At 150 mg/kg/day, the only treatment-related clinical signs consisted of increased salivation. The slight delay in vaginal opening noted compared to control animals (36 days vs33.5 days) was attributable to the slight decrease in body weight gain observed (incidental growth delay compared to controls) and the slight decrease in uterus weights seen at necropsy was considered to be incidental.

In conclusion, these results show that BCS-CN45153 interferes with pubertal development.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige powder
Lot / Batch #:	NLL 8224-10-2
Purity:	98.4% (w/w)
CAS #	Not assigned
Stability of test compound:	Until 13 March 2011

2. Vehicle and / or positive control: vehicle: 0.5% aqueous methylcellulose 400

3. Test animals:	
Species:	Rat
Strain:	Sprague Dawley Crl:CD(SD)
Age:	Female pups, 19 days of age at the start of dosing
Weight at dosing:	31.3-38.4g
Source:	[redacted]
Acclimation period:	13 days
Diet:	A04C-10 diet from [redacted], [redacted], France ad libitum
Water:	Filtered and softened tap water from the municipal water supply ad libitum
Housing:	During acclimatization, dams were housed individually with litter in Makrolon cages with laboratory-grade wood shavings other than cedar, or with corn cob. After randomization,

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	weanling females housed in groups of 3 in Makrolon cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 per hour
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN AND METHODS

1. **In life dates:** 17 November 2010-13 December 2010.

2. **Animal assignment and treatment**
All animals were examined during the acclimatization phase. On the day of randomization, the animals were weighed, and a computerized randomization procedure (XMS Path Tox V4.2.2) was used to select animals for the study from the middle of the weight range of the available animals, to ensure a similar body weight distribution among groups.

In a preliminary study, groups of immature rats were treated for 10 days at 100, 250 and 500 mg/kg bw/day. Body weight gain was decreased by 10 and 14% at 250 and 500 mg/kg bw/day relative to controls. No findings were observed at necropsy. Based on these results, the doses selected for this study were 150 and 450 mg/kg bw/day.

Groups of six animals were administered BCS-CN45153 by oral gavage for at least 20 days.

3. **Preparation and analysis of dosing solutions**
The appropriate amount of BCS-CN45153 was suspended in a 0.5% aqueous methyl-cellulose 400 solution, and stored in glass bottles at 5°C when not in use.

No analysis of the test material in vehicle was performed.

4. **Daily observations**
Animals were observed for clinical signs at least once each day starting on day 1 and every day throughout the study. Animals were also checked twice daily for moribundity and mortality throughout the study, except on weekends and public holidays when they were checked once daily.

5. **Body weight**
Body weights were measured on day 1 and daily thereafter throughout the treatment period.

6. **Vaginal opening**
Vaginal opening was recorded daily, starting on study day 10 until study day 21.

7. **Post-mortem examinations**
All animals were sacrificed approximately 24 hours after the last dose of BCS-CN88460, by exsanguination under isoflurane inhalation-induced deep anesthesia.

In all groups, the uterus was trimmed free of fat and connective tissue, and the vagina was removed from the uterus just below the cervix so that the cervix remained with the uterine body. The uteri with luminal fluid were weighed to determine wet uterine weight, then merced, blotted, and weighed again to determine blotted uterine weight.

8. **Statistics**
For body weight gain parameters, terminal body weight, absolute and relative organ weight parameters, and clinical chemistry parameters, the Bartlett test was performed to compare

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the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight and average food consumption per day, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.
If one or more group variances were equal to 0, means were compared using non-parametric procedures.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities during the study.

B. CLINICAL OBSERVATIONS

Increased salivation was noted on one or more occasions in all animals treated with BCS-CN45153, especially during the second half of the treatment period. Additionally, at 450 mg/kg bw/day, two animals showed reduced motor activity and lack of grooming on one occasion in each animal.

C. BODY WEIGHT

There was no effect on body weight or on body weight gain at either 150 mg/kg bw/day or 450 mg/kg bw/day.

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Table 5.8.1/03-1: Body weight and cumulative body weight gain in female rats administered BCS-CN45153 for 20 days

	BCS-CN88460, dose in mg/kg bw/day		
Day	**0**	**150**	**450**
1	34.0	33.8	34.5
7	61.3	58.1	59.5
14	100.7	91.1	95.7
20	136.3	126.9	133.9
Gain, days 1-20, g	102.2	93.2	99.4

D. VAGINAL OPENING

The mean age of first vaginal opening in animals administered BCS-CN45153 at 450 mg/kg bw/day was significantly reduced compared to control animals. On study day 10, the first day of vaginal opening observation, the process was already complete in four of the six females in this group. This effect was considered related to the treatment as it was correlated to a lower mean body weight at vaginal opening.

At 150 mg/kg bw/day, there was a slight delay in vaginal opening compared to control animals, but the mean body weight at vaginal opening was similar to the control group. This indicates that this delay was not a true effect on pubertal onset but was attributable to the slight decrease in body weight gain observed at this dose level.

Table 5.8.1/03-2: Mean age and body weight at vaginal opening in female rats administered BCS-CN45153 for 20 days

	BCS-CN88460, dose in mg/kg bw/day		
	0	**150**	**450**
Mean age, days	33.5	36.0	≤ 30.3
Mean body weight, g	110.0	114.8	≤ 87.9

E. NECROPSY

Mean terminal body weight was not affected by administration for 20 days of BCS-CN45153. There was also no biologically significant effect on either wet or blotted uterine weight; although uterine weights were decreased at 150 mg/kg bw/day, there was no statistical significance and no relationship to dose, so that this reduction in uterine weight was considered to be incidental.

Table 5.8.1/02-3: Terminal body weight and uterine weight in female rats administered BCS-CN45153 for 20 days

		BCS-CN88460, dose in mg/kg bw/day		
		0	**150**	**450**
Terminal body wt, g		142.7	133.5	138.8
Wet uterine weight	Absolute, mg	0.2940	0.1729	0.3381
	% body wt	0.20305	0.12925	0.25096
Blotted uterine weight	Absolute, mg	0.2439	0.1645	0.2192
	% body wt	0.16975	0.12301	0.16088

F. DEFICIENCIES

There are no deficiencies to report.

III. CONCLUSIONS

At 450 mg/kg bw/day, treatment-related clinical signs consisted of increased salivation, reduced motor activity, and lack of grooming. There was no effect on mean body weight. The mean age of and mean body weight at first vaginal opening were both significantly reduced compared to controls. No significant effect was observed on uterine weight at necropsy. At 150 mg/kg bw/day,

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the only treatment-related clinical sign was an increase in salivation on some occasions. The slight delay in vaginal opening compared to control animals was attributed to the slight decrease in body weight gain observed, and thus to an incidental growth delay compared to controls, and the slight decrease in uterine weights at necropsy was considered to be incidental.

These results show that BCS-CN45153 interferes with pubertal development in the female rat, with a NOAEL at 150 mg/kg bw/day.

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CA 5.8.2 Supplementary studies on the active substance

Table 5.8.2: Summary of MOA studies conducted with BCS-CN88460 to support mode of action in liver and thyroid

Study / Review	Mode of dosing	Test material and dose levels	Conclusions
7 day dietary study of liver and thyroid cell proliferation in the female rat [redacted], D.; 2018; M-615229-01-1	Dietary administration	BCS-CN88460 0, 30, 75, 150, 450, 800 ppm 0, 2.4, 6.0, 12, 36, 67 mg/kg bw/day	Increased liver weight, increased cell proliferation in both liver and thyroid; induction of Phase I and Phase II enzymes and gene transcripts. Conclusion: BCS-CN88460 activates the CAR and / or PXR in the female rat.
7-day dietary study of liver and thyroid cell proliferation in the female mouse [redacted], D.; 2018; M-614504-01-1	Dietary administration	BCS-CN88460 0, 50, 110, 250, 560, 1250 ppm 0, 8.87, 18.6, 43.9, 94.1, 224 mg/kg bw/day	Induction of hepatic Phase I and Phase II enzymes, increased liver weight. Conclusion: BCS-CN88460 activates the CAR and potentially the PXR in the female mouse, but does not activate either AhR or PPARalpha
28-day dietary study of hepatotoxicity and thyroid hormone concentrations in the female rat [redacted]; 2013; M-466599-01-1	Dietary administration	BCS-CN88460 0, 300, 1000 ppm 0, 26.4, 84.8 mg/kg bw/day	Increased TSH concentration, TSHß gene transcript. Increased liver weight, induction of Phase I and Phase II enzymes, gene transcripts. Conclusion: BCS-CN88460 activates the CAR and / or PXR in the female rat.
28-day dietary study of cell proliferation in the liver and thyroid of the female rat [redacted], D.; 2018; M-615222-01-1	Dietary administration	BCS-CN88460 0, 30, 75, 150, 450, 800 ppm 0, 2.4, 6.0, 12, 37, 69 mg/kg bw/day	Increased liver weight, hepatocellular hypertrophy; induction of Phase I and Phase II enzymes, gene transcripts Conclusion: BCS-CN88460 activates the CAR and / or PXR in the female rat.

During the development of BCS-CN88460, it was posited that the test item would likely act through the CAR and / or PXR to cause specific effects in the liver and thyroid. A program of mechanistic studies primarily in the rat was conducted to test this hypothesis.

In a seven-day study examining the effect of BCS-CN88460 on the hepatocellular and thyroid follicular cell proliferation, female rats were administered the test item at dietary concentrations from 30 up to 800 ppm. These concentrations were selected on the basis of results obtained in earlier

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subchronic studies as well as to provide mechanistic information on events occurring at dietary concentrations used in the rat 2-year study. The female, and not the male, rat was chosen as the subacute and subchronic studies suggested that the effects observed in liver and thyroid would be greater in females than in males. There was no effect of treatment on clinical signs, body weight, body weight gain, or food consumption in any group. There were no biologically meaningful effects on the concentrations of T3, T4, or TSH in any group. Absolute liver weight was slightly increased at the high dose of 800 ppm, and relative liver weight was statistically significantly increased at that concentration. Enlarged liver was noted at necropsy at 800 ppm, although there were no related histopathological findings in any group. In the liver, there was a dose-related increase in the incidence of centrilobular, periportal, and total cell proliferation, although the increase in centrilobular proliferation was not statistically significant. There was also an increase in total proliferative index in the thyroid. The increase in cell proliferation in both liver and thyroid was rapidly reversible after withdrawal of treatment. Total cytochrome P450, activity of BROD and PROD, and activities of UDPGT enzymes were increased in a dose-related and reversible manner. These results demonstrate that BCS-CN88460 increases specific enzyme induction and cell proliferation in the liver and thyroid in the female rat, supporting the suggestion that BCS-CN88460 acts in the rodent via a CAR-PXR mode of action which is not relevant for humans.

A similar 7-day study was conducted in the mouse, with BCS-CN88460 administered via the diet at concentrations from 50 to 1250 ppm; these concentrations were selected on the basis of results obtained in earlier subchronic studies as well as to provide mechanistic information on events occurring at dietary concentrations used in the mouse 18-month study. The female, and not the male, mouse was chosen as earlier studies suggested that the effects observed in liver and thyroid would be greater in the female than in the male. There was no effect of BCS-CN88460 administration on clinical signs, body weight or body weight gain, or food consumption. Absolute and relative liver weights were increased in a dose-related manner, although there were no histopathological findings described in any group. Centrilobular, periportal, and total hepatocellular proliferation increased, although not in a dose-related manner. There was no effect on thyroid follicular cell proliferation at any dose. Total cytochrome P450 content and the activities of PROD, BQ, and bilirubin-UDPGT were increased in a dose-related manner. These results demonstrate that BCS-CN88460 increases specific enzyme induction and cell proliferation in the liver in the female mouse, supporting the suggestion that BCS-CN88460 acts in the rodent via a CAR-PXR mode of action which is not relevant for humans.

A 28-day mechanistic study was conducted in the female rat, with BCS-CN88460 administered via the diet at concentrations of 0, 300, and 1000 ppm; these concentrations were chosen on the basis of concentrations used in the subacute and subchronic studies. As for the 7-day studies, the females were chosen as the effects observed in earlier studies suggested that the effect of BCS-CN88460 on the liver and thyroid would be greater in the female than in the male. There was no effect of BCS-CN88460 administration via the diet for 28 days on clinical signs or mortality. Body weight was unaffected by treatment, but body weight gain was slightly reduced at 1000 ppm over the course of the study. At necropsy, absolute and relative liver weights were increased, as were the activities of BROD, bilirubin-UDPGT, and T4-UDPGT. Circulating concentrations of T3 and T4 were not altered, but the concentration of TSH as well as the amount of TSHß transcripts in the pituitary were increased in a dose-related manner. These results confirmed that BCS-CN88460 acted on the rat liver and thyroid via the CAR-PXR mode of action.

A further 28-day dietary study was conducted in the female rat to determine the effect of BCS-CN88460 on cell proliferation in the liver and thyroid after subacute administration. The test item was added to the diet at concentrations ranging from 30 to 800 ppm, the same concentrations as those used in earlier studies including other mechanistic work in the rat. Mortality, clinical signs, body weight, body weight gain, and food consumption were not affected in any group. The concentrations of circulating T3 and T4 were not changed at any dose, but circulating TSH concentration was increased in a dose-related manner. Absolute and relative liver weights were increased, as was the incidence of enlarged liver and of hepatocellular hypertrophy. In the thyroid gland, the incidence of thyroid follicular cell hypertrophy was increased only at the top dose of 800 ppm. There was no statistically significant effect on proliferative index in the liver, but thyroid

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proliferative index was statistically significantly increased. Total cytochrome P450 content was not changed with treatment, however the activities of BROD and PROD were increased, as were the activities of para-nitrophenol-UDPGT and bilirubin-UDPGT. These results confirmed that BCS-CN88460 acted in the female rat via activation of the PXR receptor to produce effects in liver and thyroid.

Report:	KCA 5.8.2/02; [redacted], D.; 2018; M-615229-01-1
Title:	BCS-CN88460 - 7-day mechanistic toxicity study for liver and thyroid cell proliferation in female Wistar rats
Report No.:	SA 15054
Document No.:	M-615229-01-1
Guideline(s):	US EPA OCSPP 870.SUPP
Guideline deviation(s):	none
GLP/GEP:	**no**

Executive Summary

BCS-CN88460 was administered continuously via the diet to groups of female Wistar rats for at least 7 days at concentrations of 30, 75, 150, 450 and 800 ppm, corresponding to 2.4, 6.0, 12, 36 and 67 mg/kg bw/day. A similarly constituted group received untreated diet and acted as a control. Each group consisted of 12 female Wistar rats with the exception of the control group and the 800 ppm BCS CN88460 group, where 12 additional females were fed untreated control diet for a further one month to assess the reversibility of changes induced during the 7 day treatment period. All animals were observed for mortality and clinical signs daily, body weight and food consumption were measured weekly. A detailed physical examination was performed weekly throughout the study. Before necropsy a blood sample was collected from the aorta of each animal for hormone analyses and clinical chemistry determinations. All animals were necropsied either following at least 7 days of treatment or at the end of the recovery phase. At both sacrifice times, blood samples were taken for hormone analysis (T4, T3 and TSH) and the liver, thyroid and pituitary gland from each animal were collected. The liver was weighed and sampled for investigation of several parameters. Specifically, samples were fixed and examined microscopically. Additional slides were stained for Ki67 for cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations. The pituitary gland and small portions of the liver were frozen in liquid nitrogen and used for gene expression investigations. The remaining portions of the liver were homogenized for microsomal preparations in order to determine cytochrome P-450 and UDPGT isoenzyme profiles.

Dietary administration of BCS-CN88460 for at least 7 days to female Wistar rats at dose levels of 30, 75, 150, 450 and 800 ppm induced no mortalities or treatment-related clinical signs and no changes in body weight parameters or in food consumption.

BCS-CN88460 had no effect on terminal body weight but induced treatment-related changes in the liver and thyroid parameters investigated as follows:

Treatment phase:

At 30 ppm (equating to 2.4 mg/kg bw/day), no treatment related effects were recorded at this dose level. At 75 ppm (equating to 6.0 mg/kg bw/day), a statistically significant increase in the expression of Cyp2b1 was recorded (+363%; p≤0.01), which increased with increasing dose up to +7025% at 800 ppm. This increase in gene expression was, however, not associated with any statistically significant increase in corresponding enzyme (BROD) activity at any dose level. In addition, for the phase II gene Ugt2b1 a statistically significant increase in its expression (+79%, p≤0.05) was recorded which increased with increasing dose up to +270% at 800 ppm.
At 150 ppm (equating to 12 mg/kg bw/day), statistically significant (p≤0.01) increase were recorded for the expression of the Phase II gene Ugt1a1 (+35%) and for the gene expression of Gadd45b, a

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marker for cell proliferation (+47%) which increased with increasing dose up to respectively +329% and +126% at 800 ppm.
At 450 ppm (equating to 36 mg/kg bw/day), statistically significant increases were recorded for the expression of the Phase I gene Cyp3a23 (+1571%; $p \leq 0.01$) and for the hepatic enzyme activity UDPGT bilirubin (+76%; $p \leq 0.05$) mainly associated with increased expressions of the Phase II genes Ugt1a6 and Ugt2b1 (respectively +33% and +156%; $p \leq 0.01$).

At 800 ppm (equating to 67 mg/kg bw/day), statistically significant increases in mean liver weight relative to body weight (+9%; $p \leq 0.01$) and brain weight (+12%; $p \leq 0.05$) were recorded as well as enlarged liver in 6/12 animals which were not associated with any microscopic findings. However, increased cell proliferation was observed with the effects being more apparent in the periportal region (+98%; $p \leq 0.01$ compared to controls). Moreover, a marginal though not statistically significantly decrease (-23%; $p \leq 0.05$) in the expression of the Phase I gene Cyp4a1 was also recorded. Changes in gene expression and enzyme activity observed at the lower doses were also more marked at this top dose level. Finally, an increased level of plasma TSH level (+85%, not statistically significant) was recorded associated with an increased thyroid follicular cell proliferation (+111%; $p \leq 0.001$).

<u>Recovery phase:</u>

For the females previously treated with 800 ppm BCS-CN88460, there were no relevant changes in any of the body weight parameters during the recovery phase. A marginal statistically significant increase (+5%, $p \leq 0.05$) in food consumption was, however, recorded during the first week of the recovery phase. There was no effect on any other parameter at the end of the recovery phase.

Overall, this study demonstrates that BCS CN88460 administration for at least 7 days in the female Wistar rats induced clear and statistically significant changes in the liver (enzyme activity as well as associated molecular and cell proliferation changes) and in the thyroid gland (hormonal, cell proliferation changes). These effects were not detected after a four-week recovery phase. Overall, these data suggest that at the dietary levels tested, BCS-CN88460 appears to activate PXR more strongly than CAR in female rats.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% w/w
CAS #:	1255734-28-1
Stability of test compound:	Until 28 November 2015

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	Rat
Strain:	Wistar Rj:WI (IOPS HAN)
Age:	At least 10 weeks old at the start of dosing
Weight at dosing:	219-274g
Source:	[redacted]
Acclimation period:	At least 7 days
Diet:	A04CP1-10 powdered diet from [redacted], [redacted], France ad libitum
Water:	Filtered and softened tap water from the municipal water supply, ad libitum

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Housing:	In groups of four in suspended, stainless steel / polycarbonate cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15/hour
Photoperiod:	12 hours light

B. STUDY DESIGN AND METHODS

1. In life dates: 20 May 2015-6 July 2015

2. Animal assignment and treatment

All animals were weighed at least weekly and a detailed physical examination was performed once during the acclimatization phase. On the day of randomization, all animals were weighed. A computerized randomization procedure (Pristima System, version 6.3.2 build 17, Xybion Corp.) was used to select animals for the study from the middle of the weight range of the available animals, which ensured a similar body weight distribution among groups for each sex (i.e. within ± 20% of the mean body weight on the day of randomization). Ninety six female rats were selected for the study. Selected animals were in a weight range from 219 to 274 g on the first day of treatment.

The three dietary levels for BCS-CN88460 (30, 150 and 800 ppm) correspond to the levels selected in the rat carcinogenicity study (SA 13266; M-612739-02-1 referenced in this Summary MCA Section 5 under Point 5.5/01). In addition, two intermediate dose levels (75 and 450 ppm) were selected to refine the dose-response characterization Female rats were selected as it was the most sensitive sex for liver effects in the 28 day (SA 11308; M-464024-03-1 referenced in this Summary MCA Section 5 under Point 5.3.1/01) and in the 90 day toxicity studies in the rat (SA 12102; M-487478-02-1 referenced in this Summary MCA Section 5 under Point 5.3.1/02).

Each group consisted of 12 female rats with the exception of the control group and the highest dose of BCS-CN88460 where 12 additional females were fed control or test diet (800 ppm BCS CN88460) and were then allowed one month (at least 28 days) of recovery during which they were maintained on untreated control diet. All animals were sacrificed in the morning after the last day of treatment or after the last day of the recovery phase.

3. Diet preparation and analysis

The test item was incorporated into the diet by dry mixing to provide the required dietary concentrations. When not in use the diet formulations were stored at room temperature except for 30 ppm formulation which was stored at approximately 18°C.

The stability of the test item in the diet was demonstrated on a pre study mix at concentrations of 60 and 10000 ppm for a time which covered the period of usage and storage for the study (SA 12032; M-439130-01-1; report available upon request). The stability of the test item in the diet was checked at a concentration of 30 ppm (SA 13266; M-612739-02-1 referenced in this Summary MCA Section 5 under Point 5.5/01) for a time which covers the period of storage and usage for the current study.

The homogeneity of BCS-CN88460 in diet was verified for the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. Dietary levels of the test item were verified for each concentration. Data were recorded and analyzed using Empower 3 (Build 3471).

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4. Mortality and clinical signs

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

5. Body weight

Each animal was weighed at least weekly during the acclimatization period, on the first day of test item administration, and on study day 8. Animals which had been diet-fasted overnight were weighed before scheduled necropsy to determine terminal body weight.

6. Food consumption and compound intake

The weight of food supplied and that remaining at the end of the study was recorded for each cage, and any food spillage was noted. The mean achieved dosage in mg/kg bw/day was calculated from that data.

7. Blood sampling

On the days of necropsy, blood samples were taken from all animals in all groups by puncture of abdominal aorta. Blood was collected on EDTA (approximately 2.0 mL). Plasma was separated by centrifugation and stored at approximately 74°C + 10°C.

Determination of T3 and T4 were performed by LC-MS/MS method. The TSH analysis was determined by EIA using the Rat Pituitary Magnetic Bead Panel (Merck Milipore, code RPTMAG-86K-01).

Determination of the test item and its 2 major metabolite concentrations in plasma samples were performed as recommended in Regulation (EU) No 283/2013 setting out the data requirements for active substances.

8. Necropsy

On Study Day 8 following treatment or on Day 29 following the recovery period, all animals from all groups were sacrificed by exsanguination while under deep anesthesia by inhalation of Isoflurane (Virbac, Carros, France). Animals were not diet fasted overnight prior to sacrifice.

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

The brain, liver and thyroid gland were weighed in all animals from all groups. The organs were weighed fresh, except the thyroid glands which were weighed paired after fixation.

For each animal, the thyroid gland, two central sections taken from the median and the left lobes of the liver, the macroscopic findings and a portion of the duodenum were collected and preserved in 10% neutral buffered formalin fixative.

For each animal, a piece of both the median and the left lobes of the liver plus the pituitary gland were collected and flash frozen in liquid nitrogen and stored at approximately 74°C ± 10°C until used for gene transcript analyses by quantitative Polymerase Chain Reaction (qPCR).

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The remaining portion of the liver from each animal was homogenized for microsomal preparations.

For conventional histopathological examination, three sections of the thyroid gland, the two central sections of the liver and one piece of duodenum from all animals were processed and embedded in paraffin wax. Histological slides were prepared and stained with hematoxylin and eosin for conventional histopathological examination.

For cell proliferation assessment, an immunohistochemical staining demonstrating the detection of Ki67 nuclear protein and the determination of the labeling index was performed to assess thyroid and liver cell proliferation on all study animals. A section from a formalin-fixed paraffin-embedded block containing three thyroid sections and one duodenum sample were prepared (the duodenum has a high cell proliferation rate and serves as a positive staining control). A section from a paraffin-embedded block including two pieces of liver (left and median lobes) and one duodenum sample were also prepared. The immunohistochemical reaction included incubation with a monoclonal antibody raised against Ki67, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of the complex with the chromogen diamino-benzidine (DAB) and nuclear counterstaining with hematoxylin.

The immunohistochemical staining for Ki67 and determination of the labeling index were performed on all animals showing sufficient Ki67 staining (estimated by duodenal Ki67 labeling).

The labeling indices, expressed as the number of Ki67-positive cells per thousand in the liver and in the thyroid gland, were measured separately on multiple fields comprising in total at least 1000 cells using an image analysis system. The mean and standard deviation were calculated for each group.

9. Enzyme induction and gene transcription

At final necropsy, the remaining portions of the liver from all animals were homogenized for microsomal preparations in order to determine total cytochrome P 450 content and specific cytochrome P 450 and UDPGT isoenzyme profile to check the hepatotoxic potential of the test item. Total cytochrome P 450 content in microsomal preparations was determined by spectrophotometry (Cary Win UV version 3.0 (182)) using a reduced CO differential spectrum. A single quantification was performed for each sample.

Specific cytochrome P 450 enzymatic activities were evaluated by spectrofluorimetry (SAFAS SP2000 version 6.10.7.4) using the following substrates:

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

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

Phase II enzymatic activities were also determined by measuring UDP glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate (method adapted from Zakim and Vessey, (6). The enzymatic kinetic (disappearance of the colored 4 nitrophenol) was followed at 405 nm during 3 min. at 30°C. Three replicates from each sample were assayed.

UDPGT with bilirubin as substrate was also determined using a spectrophotometry method (Cary Win UV version 3.0 (182)) (adapted from Heirwegh et al. (7)) consisting in the

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determination of conjugated bile pigments after its conversion into azo-pigment derivatives. Absorbance was measured at 530 nm. Three replicates from each sample were assayed.

UDPGT activity with Thyroxine as substrate (Ugt-T4) was determined using radiolabelled 125I thyroxine and the formation of T4-glucuronide was evaluated by HPLC with radioflow detection.

Total RNA was used for Reverse Transcription (RT) using the Reverse transcription master mix (Fluidigm). A pre-amplification of the cDNA was performed using the Pre-Amp Master Mix (Fluidigm) and Taqman assays (Assay on demand, Applied Biosystems). The qPCR reactions were performed by using the Taqman Gene expression master mix (Applied Biosystems) with pre amplified cDNAs and Taqman assays on a BioMark machine (Fluidigm). β– microglobulin, β actin, were used as reference gene for the quantitative calculations. Milli-Q water was used as negative control.

10. Statistics

For body weight change, terminal body weight, and absolute and relative organ weight, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight, average food consumption per day, total cytochrome P450 and enzymatic activity and for hormonal parameters, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.

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II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities during the study.

B. CLINICAL OBSERVATIONS

No treatment-related clinical signs were noted during the period of the study.

C. BODY WEIGHT

Body weight and body weight gain were unaffected in either main-study or recovery-phase animals.

Table 5.8.2/02-1: Body weight and body weight gain in female rats administered BCS-CN88460 via the diet for 7 days, and in recovery-phase animals transferred to control diet for a further 29 days

	BCS-CN88460, dietary concentration in ppm					
Day	**0**	**30**	**75**	**150**	**450**	**800**
1	247.1	245.5	248.9	245.8	246.3	246.6
8	257.5	258.2	261.7	256.3	257.4	257.7
Gain, days 1-8	10.4	12.7	12.8	10.4	11.1	11.1
R-1	251.3					253.7
R-29	288.7					292.6
Gain, days R1-R29	37.4					38.9

D. FOOD CONSUMPTION AND ACHIEVED INTAKE

There was no biologically significant effect on food consumption during either the treatment or the reversibility period.

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Table 5.8.2/02-2: Mean compound intake during the treatment period, and food consumption, in g/animal/day, during the treatment and reversibility phases of the study

		BCS-CN88460, dietary concentration in ppm					
		0	30	75	150	450	800
Compound intake mg/kg bw/day		-	2.4	6.0	12	36	67
Food intake g/animal/day	8	20.56	20.46	20.94	19.84	20.85	21.54
	R8	21.84					22.96*
	R15	24.60					25.01
	R22	21.54					21.18
	R28	20.56					20.56

E. HORMONE DATA

There were no biologically meaningful effects of dietary administration of BCS-CN88460 for seven days on the concentrations of T3, T4, or TSH, nor were there any biologically significant changes observed in the reversibility animals.

Table 5.8.2/02-3: Concentrations of circulating thyroid hormones in female rats administered BCS-CN88460 for seven days

		BCS-CN88460, dietary concentration in ppm					
Day	Hormone	0	30	75	150	450	800
8	T3	0.47	0.49	0.52	0.63**	0.58*	0.53
	T4	2.59	2.51	3.09	2.35	2.70	2.33
	TSH	0.820	1.092	1.228	0.978	1.081	1.523
R29	T3	0.50					0.63**
	T4	2.76					2.83
	TSH	1.064					0.851

F. NECROPSY

There was no effect on terminal body weight or on the weight of either liver or thyroid after dietary administration of BCS-CN88460 for seven days, nor was either terminal body weight or liver or thyroid weight affected in the animals transferred to control diet for a further 29 days.

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Table 5.8.2/02-4: Terminal body weight and weight of liver and thyroid in female rats administered BCS-CN88460 via the diet for seven days, and in animals of the reversibility phase

			BCS-CN88460, dietary concentration in ppm					
Day			**0**	**30**	**75**	**150**	**450**	**800**
8	Terminal body wt, g		262.6	258.2	261.7	256.2	257.4	260.6
	Liver	Absolute wt, g	8.881	8.582	8.706	8.763	9.008	9.649
		% body wt	3.380	3.315	3.325	3.418	3.495	3.700**
		% brain wt	468.2	460.5	458.2	464.6	474.1	523.1*
	Thyroid	Absolute wt, g	0.02097	0.01755	0.02123	0.01968	0.01911	0.01884
		% body wt	0.00802	0.00683	0.00812	0.00769	0.00746	0.00724
		% brain wt	1.105	0.939	1.114	1.041	1.007	1.020
R29	Terminal body wt, g		288.7					292.6
	Liver	Absolute wt, g	8.317					8.925
		% body wt	2.878					3.047*
		% brain wt	441.9					468.2
	Thyroid	Absolute wt, g	0.01935					0.01943
		% body wt	0.00670					0.00663
		% brain wt	1.027					1.020

The incidence of enlarged liver at macroscopic examination was increased from 150 ppm after dietary administration of BCS-CN88460 for seven days. One animal in the 800 ppm reversibility group also had enlarged liver after the 29-day reversibility phase. There were no relevant histopathological findings in the 800 ppm animals.

Table 5.8.2/02-5: Incidence of enlarged liver in female rats administered BCS-CN88460 via the diet for seven days, and in animals of the reversibility phase

	BCS-CN88460, dietary concentration in ppm					
Finding	**0**	**30**	**75**	**150**	**450**	**800**
Treated animals						
Macroscopic examination, N	12	12	12	12	12	12
Liver enlarged	0	1	0	3	2	6
Reversibility phase						
Macroscopic examination, N	12					12
Liver enlarged	0					1

G. LIVER AND THYROID CELL PROLIFERATION

In both the liver and the thyroid, there was a statistically significant, dose-related increase in cell proliferation that was fully reversible. In the liver, the proliferation index was significantly

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reduced compared to controls in animals that had been administered BCS-CN88460 for seven days then transferred to control diet for a further 29 days.

Table 5.8.2/02-6: Proliferative index in liver and thyroid of female rats administered BCS-CN88460 for seven days, and in animals of the reversibility phase

		BCS-CN88460, dietary concentration in ppm					
Organ	**Location**	**0**	**30**	**75**	**150**	**450**	**800**
Treatment phase							
Liver	Centrilobular	20.16	20.54	25.41	30.01	30.03	37.19
	Periportal	34.28	25.50	37.32	38.13	48.00	67.72**
	Total	27.22	23.02	31.37	34.07	39.02	52.50**
Thyroid		23.65	22.53	31.23	25.24	37.74	49.97***
Reversibility phase							
Liver	Centrilobular	5.96					2.99
	Periportal	12.72					4.63***
	Total	9.34					3.81***
Thyroid		11.85					13.05

H. HEPATIC ENZYME INDUCTION

Total cytochrome P450 content and the activities of BROD and PROD, as well as the activities of the Phase II enzymes para-nitrophenol-, bilirubin-, and T4-UDPGT were increased with increasing dietary concentrations of BCS-CN88460. In the 800 ppm reversibility group animals the activity of EROD was increased after the 29-day reversibility phase, but none of the other Phase I and Phase II enzymes were induced.

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Table 5.8.2/02-7: Total cytochrome P450 content, and activities of specific Phase I and Phase II enzymes, after dietary administration of BCS-CN88460 in female rats

	BCS-CN88460, dietary concentration in ppm					
	0	30	75	150	450	800
Treatment phase						
Cytochrome P450 nmol/mg protein	0.882	0.815	0.839	0.917	0.893	1.048**
BROD Pmol/min/mg protein	3.873	3.583	3.421	3.844	6.809	5.444
EROD Pmol/min/mg protein	21.371	27.788	31.908**	25.095	23.578	26.288
PROD Pmol/min/mg protein	1.548	1.763	2.238	1.998	2.270	2.548
p-nitrophenol-UDPGT Nmol/min/mg protein	6.495	5.868	5.945	6.427	6.950	7.797**
Bilrubin-UDPGT Nmol/min/mg protein	0.5543	0.3913	0.4566	0.5378	0.9764*	1.4886***
T4-UDPGT Pmol/min/mg protein	0.492	0.590	0.751	0.710	0.824	1.136**
Reversibility phase						
Cytochrome P450 nmol/mg protein	0.943					0.918
BROD Pmol/min/mg protein	4.031					4.530
EROD Pmol/min/mg protein	22.059					29.803*
PROD Pmol/min/mg protein	5.146					4.992
p-nitrophenol-UDPGT Nmol/min/mg protein	6.047					5.940
Bilrubin-UDPGT Nmol/min/mg protein	0.5013					0.4239
T4-UDPGT Pmol/min/mg protein	0.930					0.850

I. GENE TRANSCRIPTION

After administration of BCS-CN88460 via the diet for seven days, specific gene transcripts related to Phase I and Phase II enzymes dependent on the CAR and / or PXR receptors were markedly increased in the livers of treated animals. The transcription of TSHβ in the pituitary was also increased in these animals. Those Phase I enzymes which are dependent on other receptors (AhR, PPARα) did not show increases in gene transcription, or only had slight increases in transcription when compared to controls. The induction in gene transcription levels was not apparent in reversibility-phase animals.

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Table 5.8.2/02-8: Transcription of specific genes in liver and pituitary in female rats administered BCS-CN88460 via the diet for seven days, and in animals of the reversibility phase

	BCS-CN88460, dietary concentration in ppm					
	0	30	75	150	450	800
Treatment phase						
Cyp1a1	1.0000	0.3017	1.1050	0.9483	1.3242	0.8042
Cyp1a2	1.0000	0.8383	0.9350	1.0458	1.0242	0.8400
Cyp2b1	1.0008	0.8817	4.6317**	6.9317**	42.8017**	71.2533**
Cyp4a1	1.0008	1.0450	1.0900	0.9983	0.9433	0.7733*
Ugt1a1	1.0000	0.9167	1.0992	1.3550**	2.9075**	4.2925**
Ugt1a6	0.9992	0.9433	0.9325	1.1167	1.3300**	1.4208**
Ugt2b1	0.9992	1.3475	1.7900*	1.7158*	2.5583**	3.7033**
TSHβ	0.9691	1.2250	1.2155	1.0875	1.3867	1.480
Reversibility phase						
Cyp1a1	0.9991					1.3217
Cyp1a2	1.0009					1.1617
Cyp2b1	1.0000					1.3842
Cyp4a1	1.0000					1.0183
Ugt1a1	1.0000					0.9325
Ugt1a6	1.0000					0.9550
Ugt2b1	1.0000					0.9925
TSHβ	0.9992					1.0150

J. DEFICIENCIES

As this study was not conducted using any guideline as a basis, there are no deficiencies to report.

III. CONCLUSIONS

Based on the above data, BCS-CN88460 produces effects on hepatic enzyme activity which are similar to those observed after administration of phenobarbital, but not similar to the results of either clofibrate (a PPAR mode-of-action positive control substance) or ß-naphthoflavone (an AhR mode-of-action positive control substance). The genes whose transcription was increased are those which are related to the hepatic enzymes induced by BCS-CN88460; genes coding for enzymes induced by clofibrate or ß-naphthoflavone were not transcribed to any greater extent in treated animals than in controls.

In summary, BCS-CN88460 acts in the liver of female rats in a manner similar to phenobarbital.

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Report:	KCA 5.8.2/03; [redacted], D.; 2018; M-614504-01-1
Title:	BCS-CN88460 - Mechanistic 7-day toxicity study for liver and thyroid cell proliferation in the C57BL/6J female mouse
Report No.:	SA 14037
Document No.:	M-614504-01-1
Guideline(s):	US EPA OCSPP 870.SUPP
Guideline deviation(s):	None
GLP/GEP:	**yes**

Executive Summary

The objective of the present study was to establish a dose response for the parameters related to CAR (constitutive androstane receptor) / PXR (pregnane X receptor) type mode of action (MOA) following 7 days treatment of BCS CN88460 in the female mouse. The second objective was to determine whether the effects observed at the highest dietary level were reversible.

The effects of different doses of BCS CN88460 on liver weight, on cell proliferation in the liver and the thyroid gland, on the hepatic total P450, PROD, BQ, UDP glucuronosyltransferase bilirubin (UGT-BL) activities and on the gene expression of in the liver were determined following continuous dietary administration for at least 7 days. In addition, the reversibility of any effects observed at the highest dose level was assessed following a recovery period of one month on untreated control diet.

BCS CN88460 was administered to female C57BL/6J mice continuously for at least 7 days in the diet at dose levels of 0, 50, 110, 250, 560 and 1250 ppm. These doses equated to 0, 8.87, 18.6, 43.9, 94.1 and 224 mg/kg/day, respectively. Each group consisted of 15 female mice with the exception of the control group and the 1250 ppm BCS CN88460 group, where 15 additional female were fed untreated control diet for a further one month to assess the reversibility of changes induced during the 7 day treatment period. Clinical observations were performed daily. Body weight and food intake were measured weekly. A detailed physical examination was performed once during the acclimatization phase and at least weekly during the dosing period. Animals were sacrificed either following at least 7 days of treatment or at the end of the recovery phase. All animals were subjected to a necropsy and the liver was weighed and sampled for investigation of several parameters. Specifically, samples were fixed and examined microscopically. Additional slides were stained for Ki67 and BrdU for cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations. A small piece of liver was flash frozen in liquid nitrogen and stored at approximately -74°C ± 10°C until used for transcript analyses by quantitative Polymerase Chain Reaction (qPCR). At both sacrifice times, the livers of 10 animals per group were homogenized for microsomal preparations in order to determine cytochrome P-450 and UDPGT isoenzyme profiles.

Dosing phase:

There were no mortalities or treatment-related clinical signs. There were no changes in body weight parameters or food consumption during the course of the study.

BCS CN88460 had no effect on terminal body weight but induced treatment-related changes in the liver parameters investigated as follows:

At 50 ppm, a statistically significant ($p \leq 0.01$) increase in PROD activity (+44%) was recorded. This increase in PROD activity was associated with significant increase in corresponding expression of Cyp2b10 (+71%), which increased with increasing dose up to +1731% at 1250 ppm. Moreover, a statistically significant ($p \leq 0.01$) increase in the expression of Cyp3a11 was recorded (+33%), which increased with increasing dose up to +614% at 1250 ppm. This increase in gene expression was, however not associated with any significant increase in corresponding enzyme (BQ) activity until 560 ppm.

At 110 ppm, statistically significant ($p \leq 0.01$) increases in enzyme PROD and UGT-BL activities (respectively +44% and 24%) were recorded. These increases in the enzyme PROD and UGT-BL

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activities were associated with significant increases in corresponding expression of Cyp2b10 (+170%; p≤0.01) and Ugt2b5 (+29%; p≤0.05). Moreover, a statistically significant (p≤0.01) increase in the expression of Cyp3a11 was recorded (+58%).

At 250 ppm, a statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight (+15%; p≤0.01; +11%; p≤0.01 respectively) were recorded. Statistically significant (p≤0.01) increases in enzyme PROD and UGT-BL activities (respectively +193% and 30%) were recorded. These increases in the enzyme activities were associated with significant (p≤0.01) increases in corresponding expression of Cyp2b10 (+372%) and Ugt2b5 (+26%). Moreover, a statistically significant (p≤0.01) increase in the expression of Cyp3a11 was recorded (+113%).

At 560 ppm, statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight (+16%; p≤0.01; +15% p≤0.01 respectively) were recorded. Statistically significant (p≤0.01) increases in enzyme Total P450, PROD, BQ and UGT-BL activities (respectively +57%, +437%, +50% and 30%) were recorded. These increases in the enzyme activities were associated with significant increases in corresponding expression of Cyp2b10 (+811%, p≤0.01), Cyp1a2 (+19%, p≤0.05), Cyp3a11 (+288%, p≤0.01), Ugt2b5 (+26%, p≤0.01) and Ugt1b1 (+27%, p≤0.01).

At 1250 ppm, statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight (+26%; p≤0.01; +27%; p≤0.01 respectively) were recorded. Statistically significant (p≤0.01) increases in enzyme Total P450, PROD, BQ and UGT-BL activities (respectively +83%, +1053%, +97% and 44%) were recorded. These increases in the enzyme activities were associated with significant (p≤0.01) increases in corresponding expression of Cyp2b10 (+1731%, p≤0.01), Cyp1a2 (+32%, p≤0.01), Cyp3a11 (+614%, p≤0.01), Ugt2b5 (+52%, p≤0.01), Ugt1b1 (+60%, p≤0.01) and Sult1d1 (+39%, p≤0.001). Moreover, a statistically significant (p≤0.01) increase in the expression of Gadd45a was recorded (+73%).

Recovery phase:

In the females initially treated with 1250 ppm BCS CN88460, all parameters were comparable with the control group.

In conclusion, this study demonstrates that BCS CN88460 administration for at least 7 days in the female C57BL/6J mouse induced clear and statistically significant changes in the liver (enzyme activity as well as associated changes in gene expression). These changes were recorded starting from 50 ppm. These effects were not detected after a four-week recovery phase. Overall, these data demonstrate that BCS-CN88460 is an inducer of Cyp2b and Cyp3a. This suggests that BCS-CN88460 is an activator of CAR (constitutive androstane receptor) and possibly PXR (pregnane X receptor) in female C57BL/6J mice. The lack of induction of the Cyp1a1 and Acox1 transcripts indicates that BCS CN88460 is not interacting with the AhR or PPARα.

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1. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006492
Purity:	94.2% w/w
CAS #	1255734-28-1
Stability of test compound:	Until 28 November 2015

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	Mouse
Strain:	C57BL/6J
Age:	Approximately 8 weeks old at start of treatment
Weight at dosing:	17.6-20.5g
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	A04CP1-10 powdered diet from [redacted], [redacted], France, ad libitum
Water:	Filtered and softened tap water from the municipal water supply, ad libitum
Housing:	Individually in suspended stainless-steel wire mesh cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 air changes per hour
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN AND METHODS

1. In life dates: 25 June 2014-7 August 2014

2. Animal assignment and treatment

All animals used were weighed and subjected to a detailed physical examination one day prior to the start of treatment. An automatic procedure was used to select animals for the study from the middle of the weight range, to ensure that all animals were within 20% of the mean body weight on the day of randomization (Pristima, version 6.3.2 built 17, Xybion Corp.).

Three dietary concentrations, namely 50, 250, and 1250 ppm, were also used in the mouse 18-month dietary study, while the intermediate concentrations of 110 and 560 ppm were selected to gain additional information between the other doses.

Groups of fifteen female mice were administered diet containing BCS-CN88460 at the stated concentrations for seven days. A further fifteen mice in the control group and fifteen mice at 1250 ppm were used in a reversibility phase, in which the mice at 1250 ppm were transferred to control diet and maintained for a further 30 days.

3. Diet preparation and analysis

BCS CN88460 was incorporated into the diet to provide the required dietary concentrations. The method of preparation was documented in the study file. The test formulations were stored at room temperature and issued to the animal unit in polyethylene containers. There was one formulation for each concentration used in the study and any unused diets were discarded at the end of each administration period.

BrdU in filtered tap water from municipal water supply was prepared during the study at 80 mg BrdU/100 ml in drinking water. There were two formulations in the study. The solutions were stored in air-tight light-resistant bottles at approximately 4°C when not in use. The unused solutions were discarded at the end of the administration period.

The homogeneity of BCS CN88460 in diet was verified at the lowest and highest concentrations. The mean values obtained from the homogeneity check were taken as

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measured concentrations. For the remaining concentrations, the dietary levels were verified for each concentration at the time of preparation.

The stability of BCS CN88460 at 20 and 10000 ppm in the diet was demonstrated in an earlier study (SA 11309; M-442490-01-1 referenced in this Summary MCA Section 5 under Point 5.3.1/02), which covered the period of storage and usage for the present study.

The concentration of BrdU in tap water was verified. The stability of BrdU in aqueous solution was demonstrated in an earlier study with a different test substance (SA 01416; available upon request), which covered the period of storage and usage for the present study with BCS-CN88460.

Data were recorded and analyzed using Empower 3 (Build 3471).

4. Mortality and clinical signs

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

5. Body weight

Each animal was weighed at least weekly during the acclimatization period, on the first day of test item administration, and on study day 8. Animals which had been diet-fasted overnight were weighed before scheduled necropsy to determine terminal body weight.

6. Food consumption and compound intake

The weight of food supplied and that remaining at the end of the study was recorded for each cage, and any food spillage was noted. The mean achieved dosage in mg/kg bw/day was calculated from that data.

7. Necropsy and histopathology

All animals from all groups were sacrificed on day 8 following dietary administration of BCS-CN88460 for seven days, or at the end of a 30-day reversibility phase. All sacrifices were performed by exsanguination under deep isoflurane inhalation anesthesia. Animals were not diet-fasted prior to necropsy. The necropsy included examination of external surfaces, all orifices, and all major body cavities and organs. Significant macroscopic abnormalities were recorded.

The liver was weighed fresh in all animals. Samples of the thyroid, two central sections taken from the median and the left lobes of the liver, and a portion of the duodenum were preserved in 10% neutral buffered formalin fixative. A further two pieces of both the median and the left lobe of the liver, and the pituitary gland, were flash-frozen in liquid nitrogen and stored at approximately -74C for qPCR analysis of gene transcripts. Finally, the remaining portion of the liver from all animals was flash-frozen in liquid nitrogen until shipment for microsomal preparation.

Two sections of the thyroid gland, two central sections of the liver, and one piece of duodenum were processed, and hematoxylin-eosin stained slides were prepared for histopathological examination. Sections were also prepared for immunochemical staining to demonstrate the incorporation of BrdU in the thyroid and liver. The immunohistochemical staining included incubation with a monoclonal antibody raised against BrdU, amplification

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with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of this complex with the chromogen diamino-benzidine (DAB), and nuclear counterstaining with hematoxylin. Moreover, immunodetection was also performed on all animals with a monoclonal antibody raised against Ki67, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of this complex with the chromogen diamino-benzidine (DAB), and nuclear counterstaining with hematoxylin.

8. Cell proliferation assessment

The labeling indices, expressed as the number of cells positive for either BrdU or Ki67 per thousand cells in the liver and in the thyroid gland, were measured separately on multiple fields containing at least a thousand cells in total. An image analysis system was used for data collection.

9. qPCR analysis

Total cytoplasmic RNA was isolated from the liver of individual control and treated animals using RNEasy mini kits. One microgram of total RNA was used for reverse transcription with a high capacity cDNA archive kit. The assay was performed in duplicate. For each gene transcript measured, a negative control condition was also used in which millipore water was used as a template rather than first-strand cDNA. The reference gene used was B-microtubulin.

10. Enzyme induction

Frozen liver fragments were processed and microsomes were prepared and stored at approximately -70C until use for determination of total cytochrome P450 content, protein concentration measurement, and measurement of specific Phase I and Phase II enzyme activities.

11. Statistics

For the main study, for body weight change, terminal body weight, and absolute and relative organ weights, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight, average food consumption per day, liver enzyme activities, cell proliferation parameters, and qPCR determinations, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root

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transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.

For the recovery phase, for body weight change, terminal body weight, and absolute and relative organ weight, the values were compared with an F test; if this F test was significant ($p \leq 0.05$), a 2-sided modified t-test was used to determine the significance of the difference between the means. If the F test was not significant ($p > 0.05$), a 2-sided T-test was used to determine the significance of the difference between the means.

For body weight, average food consumption per day, liver enzyme activities, cell proliferation parameters, and qPCR determinations, the mean and standard deviation were calculated for each group. The values were compared with an F test; if this F test was significant ($p \leq 0.05$), the data were transformed using a log transformation and an F test was again performed on the transformed data. If this F test on transformed data were significant ($p \leq 0.05$), a 2-sided modified t-test was used to determine the significance of the difference between the means. If the F test on transformed data were not significant ($p > 0.05$), a 2-sided T-test was used on the transformed data to determine the significance of the difference between the means. If the initial F test was not significant ($p > 0.05$), a 2-sided T-test was used to determine the significance of the difference between the means.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities during the study.

B. CLINICAL OBSERVATIONS

No treatment-related clinical signs were noted during either the treatment or the recovery phase.

C. BODY WEIGHT

There was no biologically significant effect of dietary administration of body weight or body weight gain in either the treatment phase or the recovery phase.

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Table 5.8.2/03-1: Mean body weight and body weight gain in female mice administered BCS-CN88460 via the diet for 7 days and in the 30-day reversibility group

	BCS-CN88460, dietary concentration in ppm					
Day	**0**	**50**	**110**	**250**	**560**	**1250**
1	19.12	19.09	19.01	19.45	19.33	19.12
7	19.37	19.17	19.39	19.87	19.41	19.06
Gain, days 1-7	0.25	0.08	0.38	0.42	0.09	-0.06
R1	19.35					19.08
R29	21.49					21.24
Gain, days R1-R29	2.15					2.16

D. FOOD CONSUMPTION AND COMPOUND INTAKE

There was no effect of BCS-CN88460 on food consumption in either the treatment-phase or the recovery phase animals.

Table 5.8.2/03-2: Mean food consumption in g/animal/day, and compound intake in mg/kg bw/day in female mice administered BCS-CN88460 via the diet

		BCS-CN88460, dietary concentration in ppm					
Day		**0**	**50**	**110**	**250**	**560**	**1250**
Compound intake		-	8.87	18.6	43.9	94.1	224
Food consumption	R8	3.39	3.40	3.28	3.49	3.26	3.42
	R8	3.53					3.41
	R15	3.83					3.57
	R22	3.90					3.73
	R29	3.94					3.95

E. TERMINAL BODY WEIGHT AND NECROPSY OBSERVATIONS

Terminal body weight considered to be related to treatment was not affected in any groups. In the 1250 ppm reversibility phase, terminal body weight was slightly reduced, however it was considered to be incidental.

Liver weight was increased in the treatment-phase animals from 250 ppm, in a dose-related manner. There was no increase in liver weight in the 1250 ppm reversibility group animals.

The incidence of enlarged liver was increased in a dose-related manner from 250 ppm in the treatment-phase animals. There were no histopathological findings in any groups.

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Table 5.8.2/03-3: Terminal body weight and weight of liver and thyroid in female mice administered BCS-CN88460 via the diet for seven days, and in animals of the reversibility phase

			BCS-CN88460, dietary concentration in ppm					
Day			**0**	**50**	**110**	**250**	**560**	**1250**
8	Terminal body wt, g		19.55	19.65	19.58	20.25	19.73	19.44
	Absolute liver wt, g		4.929	5.148	5.193	5.448**	5.653**	6.253**
	Liver, enlarged		0	1	0	3	4	8
R29	Terminal body wt, g		21.90					21.23*
	Absolute liver wt, g		5.108					5.135

F. CELL PROLIFERATION ANALYSIS

Cell proliferation assessments with BrdU labeling

In the liver, higher proliferation indices were observed from 250 ppm when compared to controls, although this increase was not dose-related.

Centrilobular, periportal, and global proliferation indices were significantly increased at 250 ppm when compared to controls. Periportal proliferation index was also significantly higher at 560 ppm when compared to controls. However, due to the observed biological and technical variabilities, no clear conclusions can be drawn from the hepatocellular proliferation data.

Table 5.8.2/03-4: Labeling index obtained with BrdU in the liver of female mice administered BCS-CN88460 via the diet for seven days

	BCS-CN88460, dietary concentration in ppm					
	0	**50**	**110**	**250**	**560**	**1250**
Centrilobular	39.97	43.74	61.56	116.56*	77.08	66.63
Periportal	36.64	38.18	57.07	102.12**	90.39*	94.62
Total	38.31	40.96	59.31	109.34**	83.74	80.62

In the thyroid, there were no treatment-related changes in cell proliferation noted at the end of the treatment period.

Table 5.8.2/03-5: Labeling index obtained with BrdU in the thyroid of female mice administered BCS-CN88460 via the diet for seven days

	BCS-CN88460, dietary concentration in ppm					
	0	**50**	**110**	**250**	**560**	**1250**
Proliferative index	14.74	9.40	14.83	21.37	15.24	11.03

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Cell proliferation assessment with Ki67 labeling

In the liver, higher periportal and global proliferation indices were observed at 1250 ppm when compared to controls, and higher periportal, centrilobular, and global proliferation indices were observed at 560 and 250 ppm compared to controls. However, as for the BrdU labeling, due to the observed biological and technical variabilities, no clear conclusions can be drawn from the hepatocellular proliferation data.

Table 5.8.2/03-6: Labeling index obtained with Ki67 in the liver of female mice administered BCS-CN88460 via the diet for seven days

	BCS-CN88460, dietary concentration in ppm					
	0	50	110	250	560	1250
Centrilobular	14.19	7.66	14.17	26.37	22.43	11.78
Periportal	9.50	7.32	10.68	19.27	19.53	17.26
Total	11.85	7.49	12.43	22.82	20.98	14.52

In the thyroid, there were no treatment-related changes in cell proliferation noted at the end of the treatment period.

Table 5.8.2/03-7: Labeling index obtained with Ki67 in the thyroid of female mice administered BCS-CN88460 via the diet for seven days

	BCS-CN88460, dietary concentration in ppm					
	0	50	110	250	560	1250
Proliferative index	6.81	4.25	6.69	11.30	8.72	6.96

G. HEPATIC ENZYME INDUCTION

There was a dose-related increase in the total cytochrome P450 concentration (from 560 ppm), and in the activities of PROD (at all doses), BQ (from 560 ppm), and bilirubin-UDPGT (from 250 ppm) in animals treated with BCS-CN88460 for seven days. In the reversibility phase animals, there was no change in enzyme induction, indicating complete reversibility of the induction seen after 7 days.

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Table 5.8.2/03-8: Hepatic Phase I and Phase II enzyme activities in female mice administered BCS-CN88460 via the diet for seven days, and in animals of the reversibility phase

	BCS-CN88460, dietary concentration in ppm					
	0	50	110	250	560	1250
Treatment phase						
Cytochrome P450 nmol/mg protein	0.30	0.28	0.36	0.35	0.47**	0.55**
PROD Pmol/min/mg protein	11.18	16.16**	19.52**	32.80**	60.09**	128.89**
BQ Nmol/min/mg protein	4.39	4.29	4.03	4.94	6.57**	8.66**
Bilrubin-UDPGT Nmol/min/mg protein	0.988	0.849	1.229*	1.283**	1.282**	1.425**
Reversibility phase						
Cytochrome P450 nmol/mg protein	0.37					0.40
PROD Pmol/min/mg protein	15.25					12.28
BQ Nmol/min/mg protein	4.88					4.79
Bilrubin-UDPGT Nmol/min/mg protein	1.256					1.087

H. qPCR ANALYSIS

There was a dose-related increase in the accumulation in the liver of transcripts which are known to be induced by activation of the CAR and / or PXR receptors, namely Cyp1a2, Cyp3a11, Ugt2b5, and Ugt1a1. The lack of induction of the Cyp1a1 transcript indicates that BCS-CN88460 does not act through the Ah receptor.

In reversibility animals, there was no increase in accumulation of any of these transcripts.

Table 5.8.2/03-9: Transcription of specific genes in the liver and pituitary of female mice administered BCS-CN88460 via the diet for seven days, and in animals of the reversibility phase

	BCS-CN88460, dietary concentration in ppm					
	0	50	110	250	560	1250
Treatment phase						
Cyp1a1	1.327	1.233	1.291	1.190	1.309	1.436
Cyp1a2	1.092	1.091	1.146	1.032	1.233*	1.372**
Cyp2b9	0.913	0.957	0.942	0.872	0.909	0.940
Cyp2b10	1.239	2.123**	3.350**	5.852**	11.292**	22.691**
Ugt2b5	0.927	1.069	1.197**	1.165**	1.437**	1.411**
Ugt1a1	0.959	1.044	1.031	1.074	1.220**	1.533**

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Table 5.8.2/03-9 continued

	BCS-CN88460, dietary concentration in ppm					
	0	50	110	250	560	1250
Reversibility phase						
Cyp1a1	0.985					1.007
Cyp1a2	0.721					0.775
Cyp2b9	0.706					0.679
Cyp2b10	1.013					1.169
Ugt2b5	0.949					0.951
Ugt1a1	0.881					0.903

I. DEFICIENCIES

As this study was not conducted using any guideline as a basis, there are no deficiencies to report.

III. CONCLUSIONS

This study demonstrates that BCS-CN88460 administration for at least 7 days in the female C57BL/6J mouse induced clear and statistically significant changes in liver enzyme activity as well as associated changes in gene expression, from a dietary concentration of 50 ppm. These effects were not observed after a 4-week recovery phase. Overall, these data demonstrate that BCS-CN88460 is an inducer of Cyp2b and Cyp3a. This suggests that BCS-CN88460 is an activator of CAR (constitutive androstane receptor) and possibly PXR (pregnane X receptor) in female C57BL/6J mice. The lack of induction of the Cyp1a1 and Acox1 transcripts indicates that BCS-CN88460 did not interact with either the AhR or PPARα.

Report: KCA 5.8.2/01; [redacted]; 2013; M-466599-01-1
Title: BCS-CN88460 - Mechanistic 28-day toxicity study in the female rat by dietary administration (hepatotoxicity and thyroid hormone investigations)
Report No.: SA 12190
Document No.: M-466599-01-1
Guideline(s): US EPA OCSPP guideline 870.SUPP
Guideline deviation(s): not applicable
GLP/GEP: **no**

Executive Summary

The objectives of this study were to better understand the histopathological changes noted in the liver and thyroid following exposure of rats with BCS-CN88460 in the previous exploratory 28-day toxicity study (SA 11308; M-464024-03-1 referenced in this Summary MCA Section 5 under Point 5.3.1/01) by providing supplementary information about its Phase I and II induced liver enzymes profile and circulating thyroid hormone levels.BCS-CN88460 (batch number: NLL 8674-19-4, a white solid, 98.6% w/w purity), a fungicide of the cypropamide family, was administered continuously via the diet to groups of female Wistar rats (10/group) for at least 28 days at concentrations of 300 and 1000 ppm, corresponding to 26.4 and 84.8 mg/kg/day, respectively. A similarly constituted group received untreated diet and acted as a control. All animals were observed for mortality and clinical signs daily, body weight and food consumption were measured weekly. A detailed physical examination was performed weekly throughout the study. At final sacrifice a blood sample was collected from the abdominal aorta of each animal for thyroid hormone measurements. All animals were necropsied and the liver was weighed. A portion of the liver and the pituitary gland from each animal were collected,

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flash frozen in liquid nitrogen and stored at approximately -74°C until use for gene transcript analyses by quantitative Polymerase Chain Reaction (qPCR). The remaining portions of the liver were homogenized for microsomal preparations in order to determine Phase I (cytochrome P 450) and II (UDPGT) isoenzymes profile.
There were no mortalities and no clinical signs observed during the course of the study at any dose level.

At 1000 ppm (equating to 84.8 mg/kg/day)
Mean body weight was unaffected, whereas mean body weight gain per day was reduced by 26% during Study Week 1 and by 15% during Study Week 4, compared to controls. The overall mean cumulated body weight gain between study days 1 and 28 was slightly reduced by 11%. Mean food consumption was reduced by 13% during the first week and by between 4 to 6% thereafter throughout the study, compared to controls.

At necropsy, thyroid hormone measurement showed a 167% increase in TSH concentration, whereas T3 and T4 concentrations were unaffected by treatment. This change in circulating TSH level was correlated with a 72% up-regulation in TSHβ gene transcripts in the pituitary gland.

Mean absolute and relative liver weights were 18% higher than the controls. Hepatotoxicity testing revealed a statistically significant increase in BROD (+57%), UDPGT bilirubin (+483%) and UDPGT T4 (+324%) activities. Genomic analysis showed that the two most up-regulated Phase I liver enzyme gene transcripts were Cyp3a3 and Cyp2b1 (+12662% and +12340%, respectively), when compared to controls. Cyp1a1 gene transcripts were also up-regulated (+459%) whereas Cyp4a1 gene transcripts were marginally down-regulated (-22%), when compared to the controls. Por (P-450 oxydoreductase) gene transcripts were for their part also up-regulated (+141%) compared to controls. Regarding phase II liver enzymes, Gstm4 (alias Gstm3) gene transcripts were the most up-regulated (+819%), when compared to controls. Ephx1, Ugt2b1 (alias Udpgtr2), Ugt1a6 and Sult2a2 gene transcripts were also significantly up-regulated by between +96 to +296%, when compared to controls.

At 300 ppm (equating to 26.4 mg/kg/day)
There were no effect on mean body weight parameters and mean food consumption.

At necropsy, thyroid hormone measurement showed a 54% increase in TSH, whereas T3 and T4 concentrations were unaffected by treatment. This change in circulating TSH level was correlated with a 16% up-regulation in TSHβ gene transcripts in the pituitary gland.

Mean absolute and relative liver weights were 8% higher than the controls. Hepatotoxicity testing revealed a statistically significant increase in BROD (+26%), UDPGT bilirubin (+131%) and UDPGT T4 (+103%) activities. Genomic analysis showed that the two most up-regulated Phase I liver enzyme gene transcripts were Cyp3a3 and Cyp2b1 (+2610% and +2544%, respectively), when compared to controls. Cyp1a1 and Cyp4a1 gene transcripts were not affected by treatment. Por gene transcripts were for their part also slightly up-regulated (+52%) compared to controls. Regarding phase II liver enzymes, Gstm4 (alias Gstm3) gene transcripts were the most up-regulated (+281%), when compared to controls. Ephx1, Ugt2b1 (alias Udpgtr2), Ugt1a6 and Sult2a2 gene transcripts were also slightly up-regulated by between +32 to +113%.

In conclusion, BCS-CN88460 administered continuously via the diet to groups of female Wistar rats for at least 28 days at concentration of 1000 ppm and to a lesser extent at 300 ppm induced BROD and UDPGT enzymatic activities together with a significant increase in selected Phase I and II liver enzyme gene transcripts, indicating that CAR/PXR receptors were indeed activated by BCS CN88460. The increase in Cyp1a1 gene transcripts observed in the high dose group could be considered as not toxicologically-relevant, since it was not translated into an increase in EROD enzymatic activities under similar test conditions in a previous 28-day toxicity study (SA 11308; M-464024-03-1 referenced in this Summary MCA Section 5 under Point 5.3.1/01). In addition to the liver changes, a

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stimulation of TSHß gene transcript in the pituitary gland and subsequent elevation in circulating TSH hormone level were noted.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	White solid
Lot / Batch #:	NLL 8674-19-4
Purity:	98.6% w/w
CAS #	1255734-28-1
Stability of test compound:	Until 11 December 2012

2. Vehicle and / or positive control: diet

3. Test animals:	
Species:	Female rat
Strain:	Wistar Rj.WI (IOPS HAN)
Age:	Approximately 7 weeks at start of treatment
Weight at dosing:	176-195g
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	A04CP1-10 powdered diet from [redacted], [redacted], France
Water:	Filtered and softened tap water from the municipal water supply ad libitum
Housing:	Individually in suspended stainless steel wire-mesh cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 changes/hour
Photoperiod:	12hours light, 12 hours dark

B. STUDY DESIGN AND METHODS

1. **In life dates:** 17 October 2012-23 November 2012

2. **Animal assignment and treatment**
   All animals used were weighed and subjected to a detailed physical examination one day prior to the start of treatment. An automatic procedure was used to select animals for the study from the middle of the weight range, to ensure that all animals were within 20% of the mean body weight on the day of randomization (Pristima, version 6.3.2 built 17, Xybion Corp.).
   The dose levels were selected based on the results of a previous 28-day toxicity study (SA 11308; M-464024-03-1 referenced in this Summary MCA Section 5 under Point 5.3.1/01) in which male and female rats were administered BCS-CN88460 via the diet at concentrations of 0, 300, 1000, and 3000 ppm. At 3000 ppm (285 mg/kg bw/day in females), liver and thyroid weights were significantly increased relative to controls, and at gross examination showed enlarged liver, dark liver, and / or prominent lobulation of the liver.At microscopic examination, treatment-related histopathological findings were observed in the liver and thyroid gland in females. At 1000 ppm (87 mg/kg bw/day in females), liver weight was moderately increased at necropsy, and enlarged and / or dark liver was noted. At microscopic examination, treatment-related findings were observed in

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the liver in female rats. At 300 ppm (26 mg/kg bw/day in females), there were no treatment-related findings in female rats.

Based on these findings, in this study groups of 10 female rats received diet containing BCS-CN88460 at 0, 300, and 1000 ppm.

3. Diet preparation and analysis

The test item was incorporated into the diet by dry mixing to provide the required dietary concentrations. When not in use, the diets were stored at room temperature. There was one formulation for each concentration in the study.

The stability of BCS-CN88460 in the diet was demonstrated in a previous study at concentrations of 60 and 10000 ppm for a time which covered the period of usage and storage for the study. The homogeneity of BCS-CN88460 in the diet was verified for both concentrations to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as the measured concentration. Dietary levels of the test item were verified for each concentration.

Results for homogeneity / concentration of BCS-CN88460 in the diet were between 94% and 97% of nominal values, thus within the in-house target range of 85-115% of nominal values. The diet mixtures were thus considered acceptable for use on the study.

4. Daily observations

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

5. Body weight

Each animal was weighed at least weekly during the acclimatization period, on the first day of test item administration, and on a weekly basis thereafter. Animals were weighed before scheduled necropsy to determine terminal body weight.

6. Food consumption and compound intake

The weight of food supplied and that remaining at the end of each food consumption period was recorded for each cage, and any food spillage was noted. The mean achieved dosage in mg/kg bw/day was calculated from that data.

7. Blood sampling and hormone measurement

At scheduled sacrifice on study days 29, 30, or 31, blood samples were taken from all animals in all groups by puncture of the abdominal aorta. Prior to bleeding the animals were anesthetized by isoflurane inhalation. Blood was collected onto clot activator for serum preparation, and serum aliquots were frozen at approximately -74C until measurement of T3, T4, and TSH by ELISA.

8. Necropsy

On study days 29, 30, or 31, all animals were sacrificed by exsanguination under deep isoflurane inhalation anesthesia. Necropsy included examination of all major organs, tissues, and body cavities. The liver and the pituitary gland collected, and the liver was weighed. A small portion of the liver was flash frozen in liquid nitrogen and stored at approximately -74C for subsequent gene transcript analysis. The remaining portions of the

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liver from all animals were homogenized for microsomal preparations in order to determine total cytochrome P450 content and the activity of specific Phase I and Phase II enzymes.

9. Enzyme induction

Total cytochrome P450 content in microsomes was determined by spectrophotometry using a reduced carbon monoxide differential spectrum. Specific cytochrome P450 activities were evaluated by spectrofluorimetry using pentoxyresorufin and benzoxyresorufin as substrates. The activity of Phase II enzymes was measured using bilirubin as a substrate with spectrophotometry detection, and using radiolabelled thyroxine as a substrate and using HPLC and radioflow detection to quantify both T4 and T4-glucuronide.

10. qPCR analysis

Frozen samples of liver and pituitary from each animal were individually ground to a powder, and total cytoplasmic RNA was isolated from individual control and treated animals using RNEasy kits. Total RNAs were quantified by spectrophotometry. Total RNA was then used for reverse transcription using a high capacity cDNA archive kit. For each gene transcript measured, a negative control condition was also used in which millipore water was used as a template rather than first-strand cDNA.

11. Statistics

For body weight change parameters, terminal body weight and absolute and relative organ weight parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight and average food consumption per day, Phase I and II enzymatic activities, hormonal parameters, and qPCR determinations, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test

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was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.
If one or more group variances were equal to 0, means were compared using non-parametric procedures.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities during the study at any dose level.

B. CLINICAL OBSERVATIONS

There were no treatment-related clinical signs observed in any dose group.

C. BODY WEIGHT

At 1000 ppm, mean body weight was not decreased by BCS-CN88460 administration. Overall cumulative body weight gain was slightly but statistically not significantly reduced. There were no effects on body weight or body weight gain at 300 ppm.

Table 5.8.2/01-1: Body weight and body weight gain in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm		
Day	**0**	**300**	**1000**
1	185.8	185.4	186.6
8	213.7	211.3	207.3
15	230.5	226.5	223.7
22	240.7	241.2	235.7
28	252.6	251.4	245.7
Gain, days 1-28	66.8	66.0	59.1

D. FOOD CONSUMPTION AND COMPOUND INTAKE

At 1000 ppm, during the first week there was a statistically significant reduction in food consumption. For the remainder of the study, food consumption was slightly but statistically not significantly reduced at this dose. There was no effect on food consumption at 300 ppm.

Table 5.8.2/01-2: Food consumption, in g/day, in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm		
Day	**0**	**300**	**1000**
8	20.53	20.00	17.87**
15	20.43	20.13	19.71
22	20.61	20.57	19.70
28	21.13	20.88	19.95
Compound intake, mg/kg bw/day	-	26.4	84.8

E. HORMONE MEASUREMENT

There was no change at either 300 or 1000 ppm in either T3 or T4 concentrations. The concentration of TSH was increased at 1000 ppm, with a tendency towards an increase also at 300 ppm.

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Table 5.8.2/01-3: Concentration of thyroid hormones in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm		
	0	300	1000
T3	0.90	0.97	0.94
T4	2.36	2.31	2.13
TSH	0.381	0.586	1.018**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

F. NECROPSY

Mean absolute and relative liver weights were increased at 1000 ppm, with a slight increase observed for relative liver weight at 300 ppm as well.

Table 5.8.2/01-4: Absolute and relative liver weight in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm		
	0	300	1000
Absolute liver wt, g	8.234	8.951	9.794**
Liver wt, % body wt	3.269	3.54*	3.865**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

G. ENZYME INDUCTION

There was no effect of treatment on the activity of PROD at either 300 or 1000 ppm. The activities of BROD, bilirubin-UDPGT and T4-UDPGT were increased in a dose-related manner at both 300 and 1000 ppm.

Table 5.8.2/01-5: Activity of specific hepatic cytochrome P450 isozymes in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm		
	0	300	1000
BROD Pmol/min/mg protein	4.34	5.49*	6.83**
PROD Pmol/min/mg protein	3.45	3.68	2.74*
Bilirubin-UDPGT Nmol/min/mg protein	0.35	0.81*	2.08***
T4-UDPGT Pmol/min/mg protein	1.03	2.09*	4.37**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

H. qPCR ANALYSIS

There was a dose-related increase in transcription of a number of Phase I and Phase II genes with increasing dietary BCS-CN88460 concentration, including those coding for cytochrome P450 1A1, 2B1, and 3A3. Although increased transcription of cyp1a1 was not expected, it is considered to be spurious given that in a similar 28-day study (referenced under Point 5.3.1/01) there was no induction of EROD activity with dietary BCS-CN88460 administration. The transcription of Por, or cytochrome P450 oxydoreductase, indicates an overall up-regulation of the cytochrome P450 mechanism. The accumulation of TSHß transcript in the pituitary was also increased.

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Table 5.8.2/01-6: Accumulation of gene transcripts for specific hepatic genes and for pitutitary thyroid stimulating hormone in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm		
	0	**300**	**1000**
Cyp1a1	1.88	2.00	10.50**
Cyp2b1	0.25	6.61	31.10**
Cyp3a3	0.77	20.87	98.27***
Cyp4a1	0.96	0.88	0.75*
Por	0.56	0.85**	1.35**
Ugt1a6	0.69	0.91	2.16***
Ugt2b1	0.65	1.15**	2.39**
Sult2a2	0.67	1.14	1.31*
Ephx1	0.72	1.53**	2.86**
Gstm4	0.48	1.83**	4.41**
TSHβ	1.09	1.26	1.87*

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

I. DEFICIENCIES

As this study was not conducted using any guideline as a basis, there are no deficiencies to report.

II. CONCLUSIONS

BCS-CN88460, administered via the diet to groups of female Wistar rats for at least 28 days, induced BROD and UDPGT enzymatic activities along with a significant increase in selected hepatic Phase I and Phase II enzyme gene transcripts, indicating that CAR and / or PXR receptors were activated by BCS-CN88460. A stimulation of the transcription of TSHß in the pituitary gland, and a subsequent elevation in circulating TSH hormone level were noted. Thus, BCS-CN88460 has a primary effect on the liver through activation of the CAR and / or PXR, and a secondary effect on the thyroid in the rat via increased UDPGT-mediated degradation of T4 and stimulation of the thyroid through increased concentration of TSH.

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Report: KCA 5.8.2/04; [redacted] D., 2018; M-615222-01-1
Title: BCS-CN88460: 28-day mechanistic toxicity study for liver and thyroid cell proliferation in female Wistar rats
Report No.: SA 15258
Document No.: M-615222-01-1
Guideline(s): US EPA OCSPP 870.3PP
Guideline deviation(s): None
GLP/GEP: **no**

Executive Summary

BCS-CN88460 was administered continuously via the diet to groups of female Wistar rats for at least 28 days at concentrations of 30, 75, 150, 450 and 800 ppm, corresponding to 2.4, 6.0, 12, 37 and 69 mg/kg/day. A similarly constituted group received untreated diet and acted as a control. Each group consisted of 12 female Wistar rats with the exception of the control group and the 800 ppm BCS-CN88460 group, where 12 additional females were fed untreated control diet for a further one month to assess the reversibility of changes induced during the 28 day treatment period. All animals were observed for mortality and clinical signs daily, body weight and food consumption were measured weekly. A detailed physical examination was performed weekly throughout the study. Before necropsy a blood sample was collected from the aorta of each animal for hormone analyses and

clinical chemistry determinations. All animals were necropsied either following at least 28 days of treatment or at the end of the recovery phase. At both sacrifice times, blood samples were taken for hormone analysis (T4, T3 and TSH) and the liver, thyroid and pituitary gland from each animal were collected. The liver was weighed and sampled for investigation of several parameters. Specifically, samples were fixed and examined microscopically. Additional slides were stained for Ki67 for cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations. The pituitary gland and small portions of the liver were frozen in liquid nitrogen and used for gene expression investigations. The remaining portions of the liver were homogenized for microsomal preparations in order to determine cytochrome P-450 and UDPGT isoenzyme profiles

Dietary administration of BCS-CN88460 for at least 28 days to female Wistar rats at dose levels of 30, 75, 150, 450 and 800 ppm induced no mortalities or treatment-related clinical signs and no changes in body weight parameters or in food consumption.

BCS-CN88460 had no effect on terminal body weight but induced treatment-related changes in the liver and thyroid parameters investigated as follows:

Treatment phase:

At 30 ppm (equating to 2.4 mg/kg/day), a statistically significant increase in mean BROD and Ugt-T4 enzymatic activities (respectively +54%; $p \leq 0.05$ and +67%; $p \leq 0.01$) was recorded at terminal sacrifice.

At 75 ppm (equating to 6.0 mg/kg/day), a statistically significant ($p \leq 0.05$) increase in the expression of Cyp3a23 was recorded, which increased with increasing dose up to +2857% at 800 ppm. This increase in gene expression was, however, not associated with any statistically significant increase in corresponding enzyme (BROD) activity at any dose level. In addition, for the phase II gene Ugt1a1 a statistically significant increase in its expression (+35%, $p \leq 0.05$) was recorded. Finally, statistically significantly ($p \leq 0.01$) increased hepatic enzyme activity was, however, only observed for Ugt-T4.

At 150 ppm (equating to 12 mg/kg/day), a statistically significant increase in mean Ugt-T4 enzymatic activity (+47%; $p \leq 0.05$) was recorded at terminal sacrifice. In addition, statistically significant ($p \leq 0.01$) increases were recorded for the expression of the Phase I genes Cyp2b1 (+985%), Cyp3a23 (+191%) and for the Phase II gene Ugt1a1 (+43%).

At 450 ppm (equating to 37 mg/kg/day), slight but statistically significant increases in mean liver weight relative to body weight (+7%; $p \leq 0.05$) was recorded. In addition, statistically significant ($p \leq 0.01$) increases were recorded for the expression of the Phase I genes Cyp2b1 (+2539%), Cyp3a23 (+1183%) and for the Phase II genes Ugt1a1 (+165%) and Ugt2b1 (+135%). A statistically significant (+60%; $p \leq 0.01$), increased expression of the marker for cell proliferation (Gadd 45b) was also observed. Increased activity of BROD (+63%; $p \leq 0.05$), UDPGT 4 nitrophenol (+20%, $p \leq 0.05$), UDPGT bilirubin (+98%, $p \leq 0.01$), and Ugt-T4 (+110%, $p \leq 0.01$) were recorded from this dose level.

Furthermore, thyroid follicular cell proliferation (+62%) were statistically significantly ($p \leq 0.01$) increased compared to the controls.

At 800 ppm (equating to 69 mg/kg/day), statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight or brain weight (range between +14% and +18%; $p \leq 0.01$ or $p \leq 0.001$) were recorded as well as enlarged liver in 10/12 animals which were associated with the centrilobular to panlobular hypertrophy observed in 5/12 females. In addition, statistically significant ($p \leq 0.01$) increases were recorded for the expression of the Phase I genes Cyp1a1 (+166%), Cyp2b1 (+10178%), Cyp3a23 (+2857%) and for the Phase II genes Ugt1a1 (+307%) and Ugt2b1 (+232%). A statistically significant (+97%; $p \leq 0.01$), increased expression of the marker for cell proliferation (Gadd45b) was also observed. Increased activity of BROD (+148%, $p \leq 0.01$), UDPGT bilirubin (+152%, $p \leq 0.01$), and Ugt-T4 (+204%, $p \leq 0.01$) were recorded from this dose level. A marginal, though statistically significant (-40%; $p \leq 0.01$), decrease in EROD enzymatic activity was also recorded. Finally, a statistically significant increase (+97%; $p \leq 0.01$) in mean TSH levels was recorded at terminal sacrifice which was associated with an increased thyroid follicular cell

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proliferation (+77%, p≤0.01) compared to the controls which were associated with the follicular cell hypertrophy observed in 6/12 females.

Recovery phase:

For the females previously treated with 800 ppm BCS-CN88460, there were no relevant changes in any of the body weight parameters during the recovery phase. A statistically significant increase (+19%, p≤0.05) in food consumption was, however, recorded during the fourth week of the recovery phase. There was no effect on terminal body weight or any organ weights at the end of the recovery phase nor were there any macroscopic or microscopic changes recorded in the liver. Marginal hepatic molecular changes were still apparent at the end of the recovery phase for genes Ugt1a1 (-14%, p≤0.05), Ugt2b1 (+52%, p≤0.05) Gadd45b (- 16%, p≤0.05) and Ccnb1 (31%, p≤0.05).

Overall, this study demonstrates that BCS CN88460 administration for at least 28 days in the female Wistar rats induced clear and statistically significant changes in the liver (enzyme activity as well as associated molecular and microscopic changes) and in the thyroid gland (hormonal, cell proliferation and microscopic changes). These effects were not detected after a four-week recovery phase. Overall, these data suggest that at the dietary levels tested, BCS-CN88460 appears to activate PXR more strongly than CAR in female rats.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige solid
Lot / Batch #:	2013-006493
Purity:	94.2% w/w
CAS #	1255734-28-1
Stability of test compound:	Until 28 November 2015
2. Vehicle and / or positive control:	diet
3. Test animals:	
Species:	Female rat
Strain:	Wistar Rj:WI (IOPS HAN)
Age:	At least 10 weeks old
Weight at dosing:	215-253g
Source:	[redacted]
Acclimation period:	At least 7 days
Diet:	A04CP1-10 powdered diet from [redacted], [redacted], France
Water:	Filtered and softened tap water from the municipal supply, ad libitum
Housing:	Four per cage in suspended stainless steel wire-mesh cages
Environmental conditions:	
Temperature:	20-24C
Humidity:	40-70%
Air changes:	10-15 changes per hour
Photoperiod:	12 hours light, 12 hours dark

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B. STUDY DESIGN AND METHODS

1. In life dates: 16 December 2015-16 February, 2016

2. Animal assignment and treatment

All animals were weighed at least weekly and a detailed physical examination was performed once during the acclimatization phase. On the day of randomization, all animals were weighed. A computerized randomization procedure (Pristima, version 7.0.0 build 22, Xybion Corp.) was used to select animals for the study from the middle of the weight range of the available animals, ensuring a similar body weight distribution among groups for each sex.

Three of the dietary concentrations used (30, 150, and 800 ppm) were those also used in the 2-year rat carcinogenicity study. Two intermediate concentrations (75 and 450 ppm) were chosen to refine the dose-response characterization. Thus, the concentrations used were 0, 30, 75, 150, 450, and 800 ppm.

Each group consisted of 12 female rats with the exception of the control group and the 800 ppm group in which an additional 12 animals per group were fed the appropriate diet for 28 days and then held for a further 28 days on untreated control diet. All animals were sacrificed in the morning after the last day of treatment or of the recovery phase.

3. Diet preparation and analysis

The test item was incorporated into the diet by dry mixing to provide the required dietary concentrations. When not in use, the diet formulations were stored at approximately -18°C (30 ppm formulation) or at room temperature. Only one formulation was prepared at each concentration in the study.

The stability of the diet was demonstrated on a pre-study mix at concentrations of 60 and 10000 ppm for a time which covered the period of storage and use for the study. The stability of the test item was also checked in another study at 30 ppm for a time which covers the period of storage and use for the current study. The homogeneity of BCS-CN88460 in the diet was verified for the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. Dietary levels of the test item were verified for each concentration.

The homogeneity and concentration data for BCS-CN88460 in rodent diet were between 85% and 107% of nominal concentrations, thus within the in-house target range of 85-115% of the nominal concentration. The diet preparations were therefore considered acceptable for use.

4. Daily observations

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

5. Body weight

Each animal was weighed at least weekly during the acclimatization phase, on the first day of test item administration, and at weekly intervals throughout the treatment period and recovery phase.

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6. Food consumption and compound intake

The weight of food supplied and that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any food spillage was noted. The weekly mean achieved dosage intake in mg/kg bw/day for each week and for weeks 1-4 was calculated from this data.

7. Blood sampling

On the day of scheduled necropsy, blood samples were taken from all animals in all groups by aorta puncture. Animals were not diet-fasted prior to bleeding. Samples were collected under deep anesthesia ensured by isoflurane inhalation. Blood was collected onto EDTA for hormone analysis and clot activator for serum clinical chemistry parameters. For hormone analysis, plasma was separated by centrifugation and stored at -74°C until shipping for hormone measurement. TSH concentration was measured by Luminex MAP technology, while T3 and T4 were measured by solid phase extraction on Oasis HLB cartridges. Total bilirubin and total cholesterol were used as indicators of compound administration and biological effect.

The concentrations of BCS-CN88460 and its two marker metabolites BCS-CX99798 and BCS-CX99799 were measured in plasma prepared from all animals.

8. Necropsy procedures

On study day 29 of the treatment or recovery phases, all animals from all groups were sacrificed by exsanguination under deep isoflurane anesthesia. Animals were not diet-fasted overnight prior to sacrifice. All animals were necropsied, with an examination of external surfaces, all orifices, and all major organs, tissues, and body cavities. The brain, liver, and thyroid gland were weighed.

Samples of the liver and thyroid gland were fixed by immersion in 10% neutral buffered formalin for histopathology and measurement of cell proliferation. The pituitary gland and a piece of the median and left lobes of the liver were collected and flash frozen in liquid nitrogen and stored at -74°C until used for qPCR. The remaining portion of the liver was homogenized for microsomal preparation.

Two sections of the thyroid gland and selected sections of the liver were processed and stained with hematoxylin-eosin for histopathological examination.

An immunohistochemical staining, to determine the presence of the Ki67 nuclear protein and thus to demonstrate thyroid and liver cell proliferation, was performed on all study animals. Sections of thyroid and liver were prepared. The immunohistochemical reaction included incubation with a monoclonal antibody raised against Ki67, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of the complex with the chromogen diamino-benzidine (DAB), and nuclear counterstaining with hematoxylin.

The labeling indices, expressed as the number of Ki67-positive cells per thousand in the liver and in the thyroid gland, were measured separately on multiple fields comprising in total at least 1000 cells using an image analysis system.

9. Liver enzyme induction and gene transcription

Liver from all animals was homogenized for microsomal preparation to determine total cytochrome P450 content and induction of specific cytochrome P450 isozymes. Total cytochrome P450 concentration was determined by spectrophotometry using a reduced CO differential spectrum.

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The activities of CYP 1A, 2B, and 3A were measured by de-alkylation of ethoxyresorufin, pentoxyresorufin, and benzoxyresorufin, respectively. Enzyme activities were measured by spectrofluorimetry over a period of 2, 5, or 7 minutes.

Phase II enzyme activities were determined by measuring the metabolism of 4-nitrophenol and bilirubin via spectrophotometry and of radiolabelled thyroxin by HPLC with radioflow detection.

10. qPCR analysis

Total RNA was used for reverse transcription. qPCR reactions were performed with Taqman gene expression methods, and ß-microglobulin and ß-actin were used as reference genes.

11. Statistics

For body weight change, terminal body weight, absolute and relative organ weights, and clinical chemistry parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight, average food consumption per day, total cytochrome P450 content and enzymatic activities, hormonal parameters, and gene transcript analysis, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$) means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.

For cell proliferation parameters, the Levene test was performed to compare the homogeneity of group variances. As the Bartlett test of homogeneity is very sensitive to non-normality of data, the Levene test was preferred. If the Levene test was not significant

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($p > 0.05$), means of the treated groups were compared to the mean of the control group using the 1-sided Dunnett test. If the Levene test was significant ($p \leq 0.05$), data were transformed using the log transformation. If the Levene test on log-transformed data were not significant ($p > 0.05$), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1-sided) on log transformed data. If the Levene test was significant ($p \leq 0.05$ even after log transformation, means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).

In the recovery phase, for body weight change, terminal body weight, and absolute and relative organ weights, the mean and standard deviation were calculated for each group and per time period for body weight change. The values were compared with an F test; if this F test was significant ($p \leq 0.05$), a 2-sided modified t-test was used to determine the significance of the difference between the means. If the F test was not significant ($p > 0.05$), a 2-sided T-test was used to determine the significance of the difference between the means.

For body weight parameters, average food consumption per day, total cytochrome P450 content and liver enzyme activities, gene transcript analysis, and hormonal parameters, the mean and standard deviation were calculated for each group. The values were compared with an F test; if this F test was significant ($p \leq 0.05$), the data were transformed using a log transformation and an F test was again performed on the transformed data. If this F test on transformed data were significant ($p \leq 0.05$), a 2-sided modified t-test was used to determine the significance of the difference between the means. If the F test on transformed data were not significant ($p > 0.05$), a 2-sided T-test was used on the transformed data to determine the significance of the difference between the means. If the initial F test was not significant ($p > 0.05$), a 2-sided T-test was used to determine the significance of the difference between the means.

For cell proliferation parameters, the F test was performed to compare the homogeneity of group variances. If the F test was not significant ($p > 0.05$), the mean of the test group was compared to the mean of the control group using the t-test (1-sided). If the F test was significant ($p \leq 0.05$), data were transformed using the log transformation. If the F test on log transformed data were not significant ($p > 0.05$), means of the test group were compared to the mean of the control group using the t-test (1-sided) on log transformed data. If the F test was significant ($p \leq 0.05$) even after log transformation, the mean of the test group was compared to the mean of the control group using the exact Mann-Whitney test (1-sided).

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities during the study.

B. CLINICAL OBSERVATIONS

No treatment-related clinical signs were observed during the study.

C. BODY WEIGHT

There were no relevant, treatment-related changes in body weight parameters during either the treatment or recovery phases.

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Table 5.8.2/04-1: Body weight and body weight gain in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm					
Day	**0**	**30**	**75**	**150**	**450**	**800**
1	232.1	235.0	232.0	232.6	234.1	230.8
15	255.2	258.8	254.1	258.7	257.3	254.0
29	276.9	284.9	278.4	281.8	277.8	273.9
Gain, 1-29	44.9	50.0	46.4	49.2	43.7	43.0
R1	297.8					275.8
R15	290.9					288.8
R29	301.4					299.7
Gain, R1-R29	21.6					23.9

D. FOOD CONSUMPTION AND COMPOUND INTAKE

There were no relevant, treatment-related effects on food consumption in any group during the study.

Table 5.8.2/04-2: Food consumption and compound intake in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm					
Day	**0**	**30**	**75**	**150**	**450**	**800**
8	21.95	21.54	21.04	21.50	23.05	25.64
15	21.66	21.60	20.45	23.54	22.14	23.06
29	19.71	20.51	19.82	20.06	20.11	18.83
R8	21.80					22.10
R15	19.38					19.67
R29	19.23					22.98*
Dose, mg/kg bw/day		2.4	6.0	12	37	69

E. CLINICAL CHEMISTRY

There was a dose-related decrease in total bilirubin in the treatment-phase animals, which became statistically significant only in the 800 ppm group. In the recovery group, total bilirubin was normal in the animals which had been administered 800 ppm BCS-CN88460 for 29 days, then transferred to control diet for a further 29 days. Total cholesterol was not affected at any dietary concentration of BCS-CN88460. These biomarkers are not indications of an adverse effect, but can be used to confirm that the compound is active in the body.

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Table 5.8.2/04-3: Concentrations of total bilirubin and of cholesterol in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm					
	0	**30**	**75**	**150**	**450**	**800**
Treatment-phase animals						
TBIL µmol/L	0.55	0.43	0.41	0.33	0.23	0.13**
CHOL mmol/L	1.878	1.871	1.971	2.074	1.835	1.822
Reversibility group						
TBIL µmol/L	0.82					0.97
CHOL mmol/L	1.913					2.029

F. HORMONE CONCENTRATIONS

In the main study animals, dietary administration of BCS-CN88460 did not change circulating T3 or T4 concentrations, but the concentration of TSH was increased in a dose-related manner. This increase was only statistically significant at 800 ppm. In the recovery animals which had been formerly administered BCS-CN88460 at 800 ppm, there was no change in the concentrations of T3, T4, or TSH at the end of the recovery phase.

Table 5.8.2/04-4: Concentrations of T3, T4, and TSH in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm					
	0	**30**	**75**	**150**	**450**	**800**
Treatment-phase animals						
T3 ng/mL	0.41	0.39	0.40	0.44	0.41	0.37
T4 µg/dL	2.21	2.24	2.17	2.49	2.64	2.27
TSH ng/ml	0.720	0.734	0.857	0.882	0.942	1.421**
Reversibility group						
T3 ng/mL	0.44					0.41
T4 µg/dL	2.25					2.25
TSH ng/ml	0.602					0.862

G. TERMINAL BODY WEIGHT AND ORGAN WEIGHT

In main-study animals, there was no effect on terminal body weight. Liver weight was statistically significantly increased at 800 ppm; although there was also a statistically significant increase in liver weight at 30 ppm, in the absence of a dose relationship and in the absence of a similar effect at 30 ppm in other studies, it is considered to be spurious and not related to treatment. There was no increase in thyroid gland weight in any group. In the reversibility animals, there was no effect of treatment on either liver or thyroid weight.

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Table 5.8.2/04-5: Terminal body weight and weight of liver and thyroid in female rats administered BCS-CN88460 via the diet for 28 days

		BCS-CN88460, dietary concentration in ppm					
		0	30	75	150	450	800
Treatment-phase animals							
Terminal body wt, g		274.1	284.7	278.4	281.8	277.7	272.1
Liver	Absolute, g	8.380	9.612**	8.777	9.049	9.125	9.839**
	% body wt	3.053	3.375**	3.154	3.212	3.280*	3.611**
	% brain wt	444.27	495.60*	461.24	468.44	471.98	508.01**
Thyroid	Absolute, g	0.01608	0.01600	0.01558	0.01475	0.01692	0.01558
	% body wt	0.00589	0.00564	0.00562	0.00524	0.00610	0.00574
	% brain wt	0.85454	0.82739	0.81895	0.76855	0.87526	0.80485
Reversibility group							
Terminal body wt, g		301.5					299.7
Liver	Absolute, g	8.349					8.764
	% body wt	2.768					2.924
	% brain wt	429.84					448.14
Thyroid	Absolute, g	0.01602					0.01763
	% body wt	0.00533					0.00588
	% brain wt	0.82553					0.89998

H. MACROSCOPIC AND MICROSCOPIC FINDINGS

In the animals of the main study, there was an increase in enlarged liver and in hepatocellular hypertrophy at 800 ppm. There were no macroscopic findings in the thyroid gland, but at histopathological examination there was an increase at 800 ppm in the incidence of thyroid follicular hypertrophy. Among the reversibility animals which had previously been administered 800 ppm, two of 12 animals still showed enlarged liver at the end of the recovery phase. No relevant histopathological findings were noted in either the liver or thyroid of recovery-phase animals.

Table 5.8.2/04-6: Macroscopic and microscopic findings in liver and thyroid in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm					
	0	30	75	150	450	800
Treatment phase						
Liver						
Examined	12	12	12	12	12	12
Enlarged	1	6	0	1	1	10
Hepatocellular hypertrophy	0	0	0	0	0	5
Thyroid						
Follicular cell hypertrophy	0	0	0	0	0	6

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Table 5.8.2/04-6 continued

	BCS-CN88460, dietary concentration in ppm					
	0	**30**	**75**	**150**	**450**	**800**
Reversibility phase						
Liver						
Examined	12	0	0	0	0	12
Enlarged	0					2
Hepatocellular hypertrophy	0					0
Thyroid						
Follicular cell hypertrophy	0					0

I. LIVER AND THYROID CELL PROLIFERATION

In the liver of animals in the main study, there was a trend towards an increase in cell proliferation at the higher dietary concentrations, although proliferation never reached statistical significance. In the thyroid, there was a statistically significant increase in cell proliferation from 450 ppm. In recovery animals, cell proliferation in the liver was statistically significantly decreased, with a slight decrease also observed in the thyroid.

Table 5.8.2/04-7: Cell proliferation in liver and thyroid in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm					
	0	**30**	**75**	**150**	**450**	**800**
Treatment phase						
Liver						
Centrilobular	9.377	11.913	8.873	9.616	12.023	12.813
Periportal	13.562	23.635	16.112	17.314	18.206	20.768
Total	11.471	17.776	12.495	13.467	15.118	16.794
Thyroid						
Total	13.137	15.527	18.896	17.008	21.263**	23.311**
Reversibility phase						
Liver						
Centrilobular	1.913					0.528*
Periportal	6.811					2.943**
Total	4.363					1.737**
Thyroid						
Total	8.605					7.109

J. LIVER ENZYME INDUCTION

In the animals of the main study, there was no increase in total cytochrome P450 content, but the activity of BROD increased significantly from 450 ppm. There was a slight increase in 4-nitrophenol-UDPGT, but the activities of both bilirubin- and T4-UDPGT increased in a dose-related, statistically significant manner. The activity of EROD decreased with increasing dose of BCS-CN88460.

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Table 5.8.2/04-8: Induction of hepatic cytochrome P450 and Phase I and Phase II enzymes in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm					
	0	30	75	150	450	800
Treatment phase						
Total P450 Nmol/mg protein	0.994	1.106	0.896	0.893	0.965	1.025
BROD Pmol/min/mg protein	2.716	4.173*	3.441	3.878	4.422*	6.737**
EROD Pmol/min/mg protein	33.672	29.272	27.039	27.573	26.711	29.323**
PROD Pmol/min/mg protein	4.149	4.227	4.060	4.790	4.738	4.784
PNP-UDPGT Nmol/min/mg protein	6.730	7.793*	6.687	6.647	8.105*	7.940
BL-UDPGT Nmol/min/mg protein	0.7711	0.8328	0.8781	0.7789	1.5236**	1.9408**
T4-UDPGT Pmol/min/mg protein	1.070	1.787**	2.102**	1.573*	2.249**	3.255**
Recovery phase						
Total P450 Nmol/mg protein	0.913					0.945
BROD Pmol/min/mg protein	2.120					3.426
EROD Pmol/min/mg protein	32.868					28.959
PROD Pmol/min/mg protein	2.729					2.415
PNP-UDPGT Nmol/min/mg protein	6.273					6.335
BL-UDPGT Nmol/min/mg protein	0.5256					0.4725
T4-UDPGT Pmol/min/mg protein	1.095					1.008

K. GENE TRANSCRIPTION

In animals of the treatment group, there was a clear, dose-related increase in the transcription of Cyp2b1, Cyp3a23, Ugt1a1, and Ugt2b1, while there was no relevant increase in the transcription of Cyp1a1 or Cyp4a1. Although transcription of Cyp1a1 was statistically significantly increased relative to controls at 800 ppm, in light of the decrease in EROD activity in this group, the increase in transcription is meaningless. In the pituitary, there was a slight increase in transcription of TSHβ. In recovery phase animals, the transcription of Ugt1a1 was statistically significantly decreased, while the transcription of Ugt2b1 was statistically significantly increased. There were also slight but statistically non-significant increases in transcription of Cyp1a1, Cyp2b1, and Cyp1a2.

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Table 5.8.2/04-9: Transcription of specific genes in the liver and pituitary in female rats administered BCS-CN88460 via the diet for 28 days

	BCS-CN88460, dietary concentration in ppm					
	0	**30**	**75**	**150**	**450**	**800**
Treatment phase						
Cyp1a1	1.00	1.142	1.182	1.477	1.364	2.659**
Cyp1a2	0.998	0.907	1.126	0.936	0.985	1.036
Cyp2b1	0.999	2.183	3.866	10.853**	26.395**	102.785**
Cyp3a23	0.993	0.800	2.061*	2.908**	12.835**	29.575**
Cyp4a1	1.001	1.022	1.207	0.907	0.962	0.750
Ugt1a1	0.999	0.946	1.255*	1.435**	2.650*	4.068**
Ugt2b1	1.000	1.253	1.106	1.436	2.351**	3.318**
TSHβ	0.999	1.062	0.961	1.039	1.028	1.188
Recovery phase						
Cyp1a1	0.999					1.921
Cyp1a2	1.000					1.090
Cyp2b1	1.001					1.271
Cyp3a23	0.994					0.798
Cyp4a1	1.000					0.894
Ugt1a1	1.000					0.857*
Ugt2b1	1.000					1.518*
TSHβ	1.007					0.924

L. DEFICIENCIES

As this study was not conducted using any guideline as a basis, there are no deficiencies to report.

III. CONCLUSIONS

Overall, this study demonstrates that BCS-CN88460 administration for 28 days in the female Wistar rat induced clear and statistically significant changes in the liver, including increases in hepatocellular proliferation, Phase I and Phase II enzyme activities, and transcription of specific genes, and in the thyroid, including changes in the concentration of TSH, in cell proliferation, and in follicular cell hypertrophy. Overall, these data demonstrate that BCS-CN88460 is an activator of PXR in the female rat.

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CA 5.8.3 Endocrine disrupting properties

Endocrine-relevant studies

Table 5.8.3: Studies conducted with Isoflucypram for endocrine endpoints

Study	NOAEL	LOAEL	Effects and conclusion
Hershberger pubertal male rat study Oral gavage dosing 0, 400, 800 mg/kg bw/day [redacted]; 2012; M-443356-01-1	800 mg/kg bw/day (highest dose tested)	> 800 mg/kg bw/day	BCS-CN88460 is neither androgenic nor anti-androgenic in the developing male rat
Uterotrophic / vaginal opening study in immature female rats Oral gavage dosing 0, 400, 800 mg/kg bw/day [redacted]; 2011; M-407181-01-1	Estrogenicity / anti-estrogenicity: 800 mg/kg bw/day (highest dose tested) Systemic toxicity: < 400 mg/kg bw/day	Estrogenicity / anti-estrogenicity: > 800 mg/kg bw/day	BCS-CN88460 is neither estrogenic nor anti-estrogenic in the developing female rat

In a Hershberger assay conducted in immature male rats, BCS-CN88460 was administered for either 10 or 30 days at 400 and / or 800 mg/kg bw/day; one 10-day treatment was administered in conjunction with administration of testosterone propionate to assess potential anti-androgenicity of the test substance. Administration of BCS-CN88460 alone for 10 days did not accelerate preputial separation in immature male rats, nor did it delay preputial separation in immature rats also administered testosterone propionate to artificially trigger preputial separation. Administration of BCS-CN88460 for 30 days did not alter the timing of preputial separation in animals allowed to mature normally. The weight of the testes and related organs and tissues were not affected by administration of BCS-CN88460 in any treatment group. Thus, BCS-CN88460 does not have either androgenic or anti-androgenic potential in the Hershberger assay.

In an uterotrophic assay conducted in immature female rats, BCS-CN88460 was administered for three or 20 days at 400 mg/kg bw/day; one 3-day treatment was administered in conjunction with estradiol benzoate to assess the potential anti-estrogenicity of the test substance. Administration of BCS-CN88460 alone for three days did not accelerate vaginal opening in immature female rats, nor did it delay vaginal opening in female rats also administered estradiol benzoate to artificially trigger vaginal opening. Administration of BCS-CN88460 for 20 days did not alter the timing of vaginal opening in animals allowed to mature normally. The weight of the uterus and related organs and tissues were not affected by administration of BCS-CN88460 in any treatment group. Thus, BCS-CN88460 does not have either estrogenic or anti-estrogenic potential in the immature rat uterotrophic assay.

Endocrine disrupting properties

In addition to the Hershberger and uterotrophic assays described above, the endocrine potential of BCS-CN88460 can be assessed on the basis of results observed in the subacute, subchronic, and chronic studies conducted in the rodent and the dog, as well as more specialized results from the 2-generation reproduction study and the two developmental toxicity studies.

In repeat-dose studies, there was no toxicologically relevant effect of BCS-CN88460 on the weight or microscopic appearance of the hypothalamus, pituitary, thymus, adrenal glands, uterus, ovaries, testes,

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seminal vesicles, or accessory sex organs and tissues. The weight, histopathology, and proliferative index of the thyroid were altered by BCS-CN88460, as were the concentration of TSH and the content of transcripts for TSHß in the pituitary in mechanistic studies. Other mechanistic studies have demonstrated that these results are secondary to enzyme induction in the rat via the CAR-PXR mode of action. As human thyroid physiology is more robust than that of the rodent, it is well-accepted that thyroid effects which are secondary to CAR-PXR-mediated liver enzyme induction and cell proliferation are not endocrine effects which are relevant for humans.

In the 2-generation reproduction study, there was no effect of BCS-CN88460 administration on any measure of fertility in either males or females, nor was fecundity affected. The sex distribution of pups was not affected, and sexual maturation of the offspring was not meaningfully affected. Sperm production, sperm assessment factors, and histopathology of the male and female reproductive tracts were not affected by BCS-CN88460 administration.

Neither the 2-generation study nor the developmental toxicity studies in rat and rabbit showed any effect of BCS-CN88460 on the developing organism which would suggest that the test item had any endocrine activity.

Thus, based on the lack of findings other than effects of thyroid which are secondary to CAR-PXR-mediated liver enzyme induction, BCS-CN88460 does not possess endocrine activity.

Report: KCA 5.8.3/01; [redacted]; 2012; M-443356-01-1
Title: BCS-CN88460 - Evaluation in the weanling rat Hershberger assay coupled with preputial separation assessment
Report No.: SA11334
Document No.: M-443356-01-1
Guideline(s): US EPA OCSPP: not applicable
Guideline deviation(s): not specified
GLP/GEP: **no**

Executive Summary

The first objective of this study was to investigate in a short-term assay, specifically the weanling rat Hershberger assay, the androgenic or anti-androgenic potential of BCS-CN88460. This was achieved by assessing potential weight changes in the androgen-dependent sex accessory tissues following oral exposure for 10 days to the test item alone or to a combination of the androgen testosterone propionate and the test item.

To investigate the potential androgenic activity of BCS-CN88460, groups of 6 weanling male Sprague Dawley rats (23 days of age) were dosed daily by oral gavage for ten days with vehicle or BCS-CN88460 at either 0 or 800 mg/kg bw/day. On the day of necropsy (Study day 11, postnatal day (PND) 33), preputial separation if any was recorded and any changes in the weights of sex accessory tissues (epididymides, ventral prostate, seminal vesicle, Cowper's glands, and levator ani / bulbocavernosus muscles) were recorded.

To screen for anti-androgenic activity of BCS-CN88460, groups of 6 weanling male Sprague Dawley rats (23 days of age) received a daily dose of testosterone propionate by subcutaneous injection as well as a daily oral gavage of either the vehicle or BCS-CN88460 at 400 or 800 mg/kg bw/day for 10 consecutive days. On the day of necropsy (study day 11, PND 33), preputial separation was recorded and any changes to the weights of the sex accessory tissues (epididymides, ventral prostate, seminal vesicle, Cowper's glands, and levator ani / bulbocavernosus muscles) were recorded.

The second objective of this study was to determine whether BCS-CN88460 has the potential to affect puberty by monitoring preputial separation during oral exposure for 30 days. For this part of the study, three additional groups of 6 male rats were dosed by oral gavage for 30 days with either vehicle

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or BCS-CN88460 at 400 and 800 mg/kg bw/day, and were monitored daily from post-natal day 33 (PND 33), corresponding to study day 11, for preputial separation. The weights of the sex accessory tissues (epididymides, ventral prostate, seminal vesicle, Cowper's glands, and levator ani / bulbocavernosus muscles), testes, and liver were recorded at necropsy.

Up to the highest dose of 800 mg/kg bw/day BCS-CN88460, there was no treatment-related effect of administration of the test item for either 10 or 30 days on preputial separation, on the weight of the testes, or on the weight of the five androgen-dependent sex accessory tissues.

When administered concurrently with testosterone propionate for 10 days, BCS-CN88460 had no effect on preputial separation or on the weight of the five androgen-dependent sex accessory tissues up to the highest dose level tested of 800 mg/kg bw/day.

At 800 mg/kg bw/day, increased salivation was noted at several occasions in 4 animals throughout the dosing period.

Androgenic activity and puberty onset: Mean body weight was reduced by a maximum of 15% throughout the study compared to controls. Mean body weight gain per day was reduced by 62% on study day 2 and by between 44 and 47% on study day 3, when compared to the controls. Thereafter, mean body weight gain per day was similar to the controls or only slightly reduced on a few occasions for group 5 only. Overall, mean absolute body weight gain was reduced by 17% and by 10% following 10 and 30 days of treatment, respectively, when compared to controls. Mean absolute liver weight was increased by 23% and 10%, and mean liver weight to terminal body weight ratio by 36% and 19% when compared to controls, following respectively 10 and 30 days of treatment.

Anti-androgenic activity: Mean body weight gain per day was reduced by 85% compared to the controls on study day 3. This effect was partially offset on study day 4, where mean body weight gain per day was increased by 42% when compared to controls. Thereafter, body weight gain per day was similar to controls or was marginally increased. Overall, mean absolute body weight gain was unaffected by treatment between study day 1 and 10. Mean absolute and relative liver weights were increased by 46% and 43% respectively when compared to controls.

At 400 mg/kg bw/day, increased salivation was observed for 3 animals throughout the dosing period.

Onset of puberty: Mean body weight was reduced by a maximum of 8% throughout the study compared to controls. Mean body weight gain per day was similar to controls, except on study days 2, 5, and 26, where it was reduced by 60%, 48%, and 41% respectively when compared to controls. Overall, mean absolute body weight gain was reduced by 10% following 30 days of treatment. Mean liver weight to terminal body weight ratio was slightly decreased by 13% when compared to controls.

Anti-androgenic activity: BCS-CN88460 had no effect on body weight parameters. Mean absolute and relative liver weights were increased by 28% and 24%, respectively, when compared to controls.

In conclusion, the data indicate that the administration of BCS-CN88460 at up to 800 mg/kg bw/day for 10 or 30 days to immature male rats had no effect on preputial separation and on sex accessory tissue weights. In addition, no anti-androgenic potential was detected under the conditions of the present study.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	White solid
Lot / Batch #:	NLL 8674-19-4
Purity:	98.6% (w/w)
CAS #	1255734-28-1
Stability of test compound:	Until 15 June 2012

2. Vehicle and / or positive control: vehicle: 0.5% aqueous methylcellulose 400
Positive control: testosterone propionate, suspended in corn oil

3. Test animals:	
Species:	Rat
Strain:	Sprague Dawley Crl:CD(SD)
Age:	Male pups, 23 days of age at the start of dosing
Weight at dosing:	51.7-69.1g
Source:	[redacted]
Acclimation period:	13 days
Diet:	Before shipment, VFR 1 diet ad libitum; after receipt, A04C-10 diet from [redacted], [redacted], France ad libitum
Water:	Filtered and softened tap water from the municipal water supply ad libitum
Housing:	During acclimatization, dams were housed individually with litter in Makrolon cages with laboratory-grade wood shavings other than cedar, or with corn cob. After randomization, weanling males housed in groups of 3 in Makrolon cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 per hour
Photoperiod:	12hours light, 12 hours dark

B. STUDY DESIGN AND METHODS

1. In life dates: 9 May 2012-21 June 2012

2. Animal assignment and treatment

All animals were examined during the acclimatization phase. On the day of randomization, the animals were weighed, and a computerized randomization procedure (Pristima version 6.3.2 build 17) was used to select animals for the study from the middle of the weight range of the available animals, to ensure a similar body weight distribution among groups.

In a preliminary study, groups of four immature male rats were treated for 10 consecutive days at 350 and 700 mg/kg bw/day with BCS-CN88460. The only treatment-related effect was a slight and transient decrease in body weight gain between study days 1 and 2. Based on those results, the doses selected were 400 (for onset of puberty and anti-androgenic activity) and 800 (for androgenic activity, onset of puberty, and anti-androgenic activity) mg/kg bw/day of BCS-CN88460.

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To determine the potential androgenic activity of BCS-CN88460, the compound was administered to six animals by oral gavage at a dose of 800 mg/kg bw/day on study days one through ten.

To determine the potential anti-androgenic activity of BCS-CN88460, groups of six animals were administered testosterone propionate at 1 mg/kg bw/day by subcutaneous injection on study days one through 10, with each injection followed immediately by oral gavage administration of BCS-CN88460 at doses of 400 or 800 mg/kg bw/day.

The effect of BCS-CN88460 on the onset of puberty was examined in groups of six animals administered the test item by oral gavage at doses of 400 or 800 mg/kg bw/day on study days one through thirty.

3. **Preparation and analysis of dosing solutions**

The appropriate amount of BCS-CN88460 was suspended in a 0.5% aqueous methylcellulose 400 solution, and stored in glass bottles at 5°C when not in use. Testosterone propionate was suspended in corn oil, and stored in glass bottles at room temperature.

The stability of BCS-CN88460 suspensions at concentrations of 0.5 and 150 g/L was checked before the start of the study, and was found to be stable over at least 27 days in storage conditions similar to those of the current study.

The homogeneity of the BCS-CN88460 formulation was checked using the first set of dose formulations to demonstrate adequate formulation procedure. Mean values obtained from the homogeneity check were reported as the measured concentrations. The concentration of the second set of dose formulations was also determined.

As homogeneity and concentration of BCS-CN88460 was found to be 92-106% of nominal concentrations, and thus within the in-house target range of 90-110%, the concentrations were acceptable for use.

4. **Daily observations**

Animals were observed for clinical signs at least once each day starting on day 1 and every day throughout the study. Animals were also checked twice daily for moribundity and mortality throughout the study, except on weekends and public holidays when they were checked once daily.

5. **Body weight**

Body weights were measured on day 1 and daily thereafter throughout the treatment period.

6. **Preputial separation**

Animals monitored for androgenic or anti-androgenic activity of BCS-CN88460 were checked for preputial separation on the day of necropsy. Those animals used for examination of the effect of BCS-CN88460 on the onset of puberty were checked for preputial separation from study day 11 (post-natal day 33) and every day throughout the study until necropsy on post-natal day 53). The status of preputial separation was recorded at approximately the same time each day. Observation of individual animals for preputial separation stopped once complete preputial separation was recorded for 2 consecutive days. The age at preputial separation is the first day on which the animal shows any stage of preputial separation, whether partial or complete, while the age at complete preputial separation is the first day on which complete preputial separation was observed. In cases where either partial or complete preputial separation had not occurred prior to necropsy, age

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at necropsy plus one day and weight on the day of necropsy were used when calculating the group means.

7. Post-mortem examinations

All animals were sacrificed approximately 24 hours after the last dose of BCS-CN88460, by exsanguination under isoflurane inhalation-induced deep anesthesia.

At necropsy, for animals monitored for androgenic or anti-androgenic activity of BCS-CN88460, the ventral prostate, epididymides, seminal vesicles with coagulating glands, levator ani / bulbocavernosus muscles, and Cowper's glands were dissected and weighed fresh.

For animals monitored for the onset of puberty, the testes, ventral prostate, epididymides, seminal vesicles with coagulating glands, levator ani / bulbocavernosus muscles, and Cowper's glands were dissected and weighed fresh.

8. Statistics

In the androgenicity section of the study, for body weight change, terminal body weight, and absolute and relative organ weight parameters, the mean and standard deviation were calculated for each group and per time period for body weight change. The values were compared with an F test, if this F test was significant ($p \leq 0.05$) a 2-sided modified t-test was used to determine the significance of the difference between the means. If the F test was not significant ($p > 0.05$), a 2-sided T-test was used to determine the significance of the difference between the means.

For body weight parameters, the mean and standard deviation were calculated for each group. The values were compared with an F test, if this F test was significant ($p \leq 0.05$), the data were transformed using a log transformation and an F test was again performed on the transformed data. If this F test on transformed data were significant ($p \leq 0.05$), a 2-sided modified t-test was used to determine the significance of the difference between the means. If the F test on transformed data were not significant ($p > 0.05$), a 2-sided T-test was used on the transformed data to determine the significance of the difference between the means. If the initial F test was not significant ($p > 0.05$), a 2-sided T-test was used to determine the significance of the difference between the means.

In the anti-androgenicity section of the study, for body weight change, terminal body weight, and absolute and relative organ weight parameters, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight parameters, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the

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control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test; if this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.
If one or more group variances were equal to 0, means were compared using non-parametric procedures.

II. RESULTS AND DISCUSSION

A. MORTALITY

One animal at 800 mg/kg bw/day was found dead on study day 3. This animal's death was attributed to an error in gavage, as macroscopic findings at necropsy included foam in the trachea and pink stains on the lungs.

One further animal at 800 mg/kg bw/day was found dead on study day 5, while an animal at 400 mg/kg bw/day was found dead on study day 8. No macroscopic examination was conducted and thus no cause of death can be attributed. It is not considered that these deaths were related to oral gavage administration of BCS-CN88460.

B. CLINICAL OBSERVATIONS

Increased salivation was observed at both 400 and 800 mg/kg bw/day in several animals. No other clinical signs were observed.

C. BODY WEIGHT

In animals monitored for androgenic activity of BCS-CN88460, administration of the test item at 800 mg/kg bw/day for 10 days decreased body weight at several occasions compared to control. At the end of the study, mean body weight gain was slightly reduced compared to control.

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Table 5.8.3/01-1: Body weight and cumulative body weight gain in immature male rats administered BCS-CN88460 via oral gavage for ten days

	BCS-CN88460, dose in mg/kg bw/day			
	Body weight, g		Cumulative body weight gain, g	
Day	0	800	0	800
1	61.10	59.83		
2	66.35	61.82	5.25	1.98***
3	72.25	64.92*	11.15	5.08***
4	77.68	72.28	16.58	12.45**
5	83.47	76.52	23.37	16.68***
6	92.08	82.26*	30.98	23.86**
7	98.42	88.24*	37.32	29.84**
8	106.12	94.84*	45.02	36.44**
9	112.78	100.76*	51.68	42.36**
10	120.87	108.16*	59.77	49.76*

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

In the group examined for an anti-androgenic effect of BCS-CN88460, there was no effect of 400 mg/kg bw/day BCS-CN88460 and 1 mg/kg bw/day testosterone propionate on body weight. At 800 mg/kg bw/day of BCS-CN88460 and 1 mg/kg bw/day testosterone propionate, body weight gain was reduced on study day 3, but overall there was no effect on body weight gain.

Table 5.8.3/01-2: Body weight and cumulative body weight gain in immature male rats administered BCS-CN88460 via oral gavage and testosterone propionate at 1 mg/kg bw/day by subcutaneous injection, for ten days

	BCS-CN88460, dose in mg/kg bw/day					
	Body weight, g			Cumulative body weight gain, g		
Day	0	400	800	0	400	800
1	59.47	60.45	60.63			
2	63.45	63.63	63.57	3.98	3.18	2.93
3	70.15	68.87	64.57	10.68	8.42	3.93**
4	75.87	75.55	71.46	16.40	15.10	11.58*
5	82.27	81.32	78.28	22.80	20.87	18.40
6	90.62	90.15	86.24	31.15	29.70	26.36
7	97.23	97.42	94.12	37.77	36.97	34.24
8	105.28	104.77	102.80	45.82	44.32	42.92
9	113.27	113.73	109.96	53.80	53.28	50.08
10	122.05	122.43	120.58	62.58	61.98	60.70

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

In animals monitored for the onset of puberty, at 800 mg/kg bw/day body weight and body weight gain were slightly reduced compared to control animals. There was no effect of BCS-CN88460 administration on body weight parameters at 400 mg/kg bw/day.

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Table 5.8.3/01-3: Body weight and cumulative body weight gain in immature male rats administered BCS-CN88460 by oral gavage for thirty days

	BCS-CN88460, dose in mg/kg bw/day					
	Body weight, g			Cumulative body weight gain, g		
Day	0	400	800	0	400	800
1	60.80	60.32	61.07			
7	100.63	92.45	93.17	39.83	32.85*	31.38**
14	162.53	153.14	152.52	101.73	92.94	91.45
21	229.15	214.44	212.52	168.35	154.24	151.45
30	314.73	288.20	290.30	253.93	228.00	229.23

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

D. PREPUTIAL SEPARATION

In animals administered BCS-CN88460 at 800 mg/kg bw/day on postnatal days 23 through 32, there was no preputial separation noted in either the control or the treated group. This indicates that BCS-CN88460 did not act as an androgen in the immature male rat.

In animals administered BCS-CN88460 at 400 or 800 mg/kg bw/day as well as testosterone propionate at 1 mg/kg bw/day on postnatal days 23 through 32, preputial separation was noted in all animals of all groups. This indicates that BCS-CN88460 did not act as an anti-androgen in the immature male rat treated with testosterone propionate.

There was no effect of oral gavage administration of BCS-CN88460 at either 400 or 800 mg/kg bw/day for 30 days on the observation of preputial separation. There was a very slight delay in preputial separation in 2 of 6 animals in the 800 mg/kg bw/day group, in which complete preputial separation had not occurred prior to necropsy. Since all other individual values for age and for weight at complete preputial separation were within the historical control data range, this effect was not considered to be treatment related.

Table 5.8.3/01-4: Age and body weight at the observation of partial and complete preputial separation in male rats administered BCS-CN88460 by oral gavage for thirty days

		BCS-CN88460, dose in mg/kg bw/day		
		0	400	800
Partial separation	Mean age, days	40.83 ± 1.33	42.80 ± 0.84	41.50 ± 1.76
	Mean weight, g	208.45 ± 20.27	212.30 ± 10.17	197.62 ± 10.92
Complete separation	Mean age, days	48.00 ± 2.45	49.00 ± 2.83	50.50 ± 3.08
	Mean weight, g	276.90 ± 33.32	264.22 ± 10.20	271.97 ± 7.80

E. NECROPSY

In animals tested for the androgenic effect of BCS-CN88460 at 800 mg/kg bw/day, there was no effect of treatment on the weight of the five androgen-dependent sex accessory tissues after 10 days treatment with BCS-CN88460. Terminal body weight was slightly decreased in the treated group, while both absolute and relative liver weight were increased compared to controls. Similarly, in animals administered BCS-CN88460 at 400 or 800 mg/kg bw/day in conjunction with 1 mg/kg bw/day of testosterone propionate, to examine the anti-androgenic effect of BCS-CN88460, there was no effect on the weight of the five androgen-dependent sex accessory tissues. Terminal body weight was also unaffected in these animals.

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Table 5.8.3/01-5: Terminal body weight and weight of specific organs in immature male rats administered BCS-CN88460, either with or without testosterone propionate (TP), for 10 days

	BCS-CN88460, dose in mg/kg bw/day				
	BCS-CN88460 alone		With testosterone propionate		
	0	800	0 + TP	400 + TP	800 + TP
Terminal body wt, g	130.88	118.74*	130.68	135.57	133.94
Liver, g	5.70	7.03**	5.59	7.16*	8.14**
Epididymides, mg	120.32	115.84	157.05	164.72	156.06
Ventral prostate, mg	67.80	62.52	118.38	130.20	121.44
Seminal vesicles, mg	40.65	40.34	227.25	232.40	240.08
Cowper's glands, mg	6.02	6.62	23.75	23.50	24.30
LABC, mg	136.65	124.20	275.13	283.52	284.80

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

In animals monitored for the onset of puberty after administration of BCS-CN88460 at either 400 or 800 mg/kg bw/day for 30days, there was a slight but not statistically significant decrease in terminal body weight, at 800 mg/kg bw/day an increase in absolute and relative liver weight, but no increase at either dose in the weight of either the testes or the androgen-dependent sex accessory tissues.

Table 5.8.3/01-6: Terminal body weight and weight of specific organs in male rats administered BCS-CN88460 for 30 days

	BCS-CN88460, dose in mg/kg bw/day		
	0	400	800
Terminal body wt, g	324.67	298.82	297.72
Liver wt, g	14.76	15.37	16.19
Testis, g	2.715	2.684	2.601
Epididymides, mg	472.23	454.66	429.25
Ventral prostate, mg	292.52	231.14*	236.75*
Seminal vesicles, mg	666.37	509.54	552.25
Cowper's glands, mg	45.75	43.54	41.37
LABC, mg	706.62	577.64	619.23

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

F. DEFICIENCIES

There are no deficiencies to report.

III. CONCLUSIONS

The administration of BCS-CN88460 for 10 days, either with or without testosterone propionate, at either 400 or 800 mg/kg bw/day, did not affect either preputial separation or the weight of androgen-sensitive organs or sex accessory tissues. Additionally, the administration of BCS-CN88460 for 30 days at either 400 or 800 mg/kg bw/day had no effect on the timing of preputial separation or the weight of androgen-sensitive organs or sex accessory tissues. Based on these results, BCS-CN88460 does not have either an androgenic or an anti-androgenic activity.

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Report:	KCA 5.8.3/02; [redacted]; 2011; M-407181-01-1
Title:	BCS-CN88460 - Evaluation in the immature rat uterotrophic assay coupled with vaginal opening
Report No.:	SA 10453
Document No.:	M-407181-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Executive Summary

The objectives of this study were to investigate in the immature rat uterotrophic assay the potential of BCS-CN88460 to interfere with uterine growth or to modify the timing of vaginal opening.

In order to identify potential estrogenic effects on uterin growth, groups of 6 immature female Sprague Dawley rats (19 days old) were dosed daily by oral gavage for 3 days with either the vehicle or BCS-CN88460 at 400 or 800 mg/kg bw/day. At 24 hours after the end of the dosing period, vaginal opening was assessed and uterine weight was recorded. A separate group was administered estradiol benzoate as a positive control for the induction of an uterotrophic response.

To identify pubertal effects, additional groups of 6 immature female Sprague Dawley rats (19 days old) were dosed on a daily basis by oral gavage for 20 days with either the vehicle or with BCS-CN88460 at 400 or 800 mg/kg bw/day. The day of vaginal opening was recorded from day 10 onwards. The uterine and liver weights were recorded 24 hours after the end of the dosing period.

Three days treatment:

At 800 mg/kg bw/day, the maximum tolerated dose was exceeded, as mortality, severe clinical signs, and marked effects on body weight parameters were observed.

Specifically, one animal in this group was sacrificed prematurely on study day 3 due to severe clinical signs (piloerection, reduced motor activity, prostration, and wasted appearance) and a body weight loss of 7 g. In animals surviving to terminal sacrifice, one case of reduced motor activity was noted on study day 3. Mean body weight was lower than the controls by 18% on study day 2 and by 23% on study day 3. Overall, there was a mean cumulative body weight loss of 1.3 g compared to a gain of 8 g in the controls.

Vaginal opening on study day 4 was not examined in this group because the maximum tolerated dose was exceeded.

At necropsy, mean terminal body weight was 19% lower than the controls, while mean uterus weights were not affected by the treatment.

At 400 mg/kg bw/day, there were neither mortalities, nor clinical signs observed. Mean body weight was lower than the controls by 11% on study day 2 and by 9% on study day 3. Overall mean cumulative body weight gain between study days 1 and 3 was reduced by 42% compared to the controls.

No vaginal opening was noted prior to necropsy on study day 4.

At necropsy, mean terminal body weight was 9% lower than the controls, while mean uterus weights were not affected by the treatment.

Twenty days treatment:

At 800 mg/kg bw/day, the maximum tolerated dose was exceeded as mortality, severe clinical signs, and marked effects on body weight parameters were observed.

One animal in this group was found dead on study day 3 after showing severe clinical signs (piloerection, reduced motor activity, uncoordinated movements) and a weight loss of 5.7 g prior to death. A second animal was sacrificed prematurely on study day 4 due to severe clinical signs (piloerection, general pallor, reduced motor activity, hunched posture, and wasted appearance). A body weight loss of 7.7 g was noted for this animal on study day 4. In animals surviving to terminal sacrifice, one case of reduced motor activity and wasted appearance was noted on study day 3. Mean

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body weight was lower than the controls by between 6 and 22% throughout the study period, the effect being most pronounced on study days 3 and 4. Overall mean cumulative body weight gain between study days 1 and 30 was reduced by 10% compared to the controls.
Mean age and mean body weight at vaginal opening were not recorded in this group since the maximum tolerated dose was exceeded.
At necropsy, mean terminal body weight was 7% lower than the controls. Mean liver weights were significantly increased by 21-30% compared to the controls, while mean uterine weights were not affected by the treatment.

At 400 mg/kg bw/day, there were neither mortalities, nor treatment-related clinical signs observed throughout the study. Mean body weight was transiently lower than the controls by 7% on study days 2 and 3, and was similar to the controls at the end of the treatment period. Overall mean cumulative body weigh gain between study days 1 and 20 was similar to the controls.
There were no treatment-related effects on the mean age or mean weight at vaginal opening.
At necropsy, mean terminal body weight was similar to the controls. Mean liver weights were significantly increased by 24 to 27% compared to the controls, while mean uterus weights were not affected by treatment.

In conclusion, BCS-CN88460 had no effect on either vaginal opening date or on uterine weight in immature female rats administered the test item for either 3 or 20 days at 400 mg/kg bw/day. The maximum tolerated dose was exceeded at 800 mg/kg bw/day.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:	
Description	Beige powder
Lot / Batch #:	LIGD563-1-2
Purity:	93.6% (w/w)
CAS #	1255734-28-1
Stability of test compound:	Until 21 July 2011

2. Vehicle and / or positive control: vehicle: 0.5% aqueous methylcellulose 400
Positive control: estradiol benzoate, suspended in 0.5% aqueous methycellulose 400

3. Test animals:	
Species:	Rat
Strain:	Sprague Dawley Crl:CD(SD)
Age:	Female pups 19 days of age at the start of dosing
Weight at dosing:	32.2-47.6g
Source:	[redacted]
Acclimation period:	13 days
Diet:	A04C-10 diet from [redacted], [redacted], France ad libitum
Water:	Filtered and softened tap water from the municipal water supply ad libitum
Housing:	During acclimatization, dams were housed individually with litter in Makrolon cages with laboratory-grade wood shavings other than cedar, or with corn cob. After randomization, weanling females housed in groups of 6 in Makrolon cages until study day 11, then in groups of 3 in Makrolon cages

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Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 per hour
Photoperiod:	12hours light, 12 hours dark

B. STUDY DESIGN AND METHODS

1. **In life dates:** 22 December 2010-24 January 2011

2. **Animal assignment and treatment**
All animals were examined during the acclimatization phase. On the day of randomization, the animals were weighed, and a computerized randomization procedure (XMS Path Tox V4.2.2) was used to select animals for the study from the middle of the weight range of the available animals, to ensure a similar body weight distribution among groups.

In a preliminary study, groups of immature rats were administered BCS-CN88460 for 10 days at 200, 400, and 800 mg/kg bw/day. In that study, there was a slight mean body weight loss on study day 2 at 800 mg/kg bw/day. One animal was found dead on study day 4 at 800 mg/kg bw/day, but there had been no preceding clinical signs and a cause of death could not be established. No other treatment-related changes were noted, thus the doses selected for the definitive uterotrophic assay were 400 and 800 mg/kg bw/day.

Groups of six animals were administered BCS-CN88460 by oral gavage for either three days or 20 days. A further group of six animals were administered estradiol benzoate at 0.08 mg/kg bw/day for three days as a positive control for estrogenic effects.

3. **Preparation and analysis of dosing solutions**
The appropriate amount of BCS-CN88460 or estradiol benzoate was suspended in a 0.5% aqueous methylcellulose 400 solution, and stored in glass bottles at 5°C when not in use.

The stability of BCS-CN88460 suspensions at concentrations of 0.5 and 150 g/L was checked before the start of the study, and was found to be stable over at least 27 days in storage conditions similar to those of the current study.

No analysis of the solutions of either BCS-CN88460 or estradiol benzoate was performed.

4. **Daily observations**
Animals were observed for clinical signs at least once each day starting on day 1 and every day throughout the study. Animals were also checked twice daily for moribundity and mortality throughout the study, except on weekends and public holidays when they were checked once daily.

5. **Body weight**
Body weights were measured on day 1 and daily thereafter throughout the treatment period.

6. **Vaginal opening**
Animals were checked for vaginal opening on study day 4 (in animals treated for three days) or daily starting on study day 10 until study day 21 (in animals treated for 20 days. If vaginal opening had not occurred by the time of necropsy (i.e. post-natal day 39), the age at necropsy plus one day (i.e. post-natal day 40) was used for those animals when calculating the mean.

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7. Post-mortem examinations

All animals were sacrificed approximately 24 hours after the last dose of BCS-CN88460, by exsanguination under isoflurane inhalation-induced deep anesthesia.

The liver from animals treated for 20 days was weighed fresh. In all groups, the uterus was trimmed free of fat and connective tissue, and the vagina was removed from the uterus just below the cervix so that the cervix remained with the uterine body. The uteri with luminal fluid were weighed to determine wet uterine weight, then pierced, blotted, and weighed again to determine blotted uterine weight.

8. Statistics

For animals administered BCS-CN88460 for body weight change, terminal body weight, and absolute and relative organ weights, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test.

For body weight, mean and standard deviation were calculated for each group. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p > 0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant ($p \leq 0.05$), data were transformed using the log transformation or square root transformation as appropriate. If the Bartlett test on log-transformed data were not significant ($p > 0.05$), means were compared using the ANOVA on transformed data. If the ANOVA on transformed data were not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log-transformed data were significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on transformed data. If the Bartlett test was significant ($p \leq 0.05$) even after log transformation, group means were compared using the Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p > 0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p \leq 0.05$), means of the exposed groups were compared to the mean of the control group using the two-sided Dunn test. For urine pH, the group means were compared by the Kruskal-Wallis test. If this test was significant ($p \leq 0.05$), a two-sided Dunn test was conducted to compare the means of the exposed groups to the mean of the control group. If the Kruskal-Wallis test was negative ($p > 0.05$), no further analysis was done.
If one or more group variances were equal to 0, means were compared using non-parametric procedures.

For animals administered estradiol benzoate, for body weight change, terminal body weight, and absolute and relative organ weights, the mean and standard deviation were calculated for each group and per time period for body weight change. The values were compared with an F test; if this F test was significant ($p \leq 0.05$), a 2-sided modified t-test was used to determine the significance of the difference between the means. If the F test was not

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significant ($p > 0.05$), a 2-sided T-test was used to determine the significance of the difference between the means.

For body weight parameters, the mean and standard deviation were calculated for each group. The values were compared with an F test; if this F test was significant ($p \leq 0.05$), the data were transformed using a log transformation and an F test was again performed on the transformed data. If this F test on transformed data were significant ($p \leq 0.05$), a 2-sided modified t-test was used to determine the significance of the difference between the means. If the F test on transformed data were not significant ($p > 0.05$), a 2-sided T-test was used on the transformed data to determine the significance of the difference between the means. If the initial F test was not significant ($p > 0.05$), a 2-sided T-test was used to determine the significance of the difference between the means.

II. RESULTS AND DISCUSSION

A. MORTALITY

In the 800 mg/kg bw/day group which was treated for three days, one animal was sacrificed prematurely on study day 3 due to severe clinical signs of reduced motor activity, prostration, and wasted appearance. A body weight loss of 7g was noted for this animal on study day 3. There were no other mortalities among animals treated for three days.

In the 800 mg/kg bw/day group which was treated for 20 days, one animal was found dead on study day 3. This animal had showed severe clinical signs of piloerection, reduced motor activity, and uncoordinated movements, and had a 5.7 g body weight loss prior to death. A second animal in this group was sacrificed prematurely on study day 4 due to severe clinical signs of piloerection, general pallor, reduced motor activity, hunched posture, and wasted appearance, as well as a body weight loss on study day 4 of 7.7 g. There were no other mortalities among animals treated for 20 days.

B. CLINICAL OBSERVATIONS

The only clinical signs noted in animals surviving until the end of the study were in the 800 mg/kg bw/day groups; one animal treated for three days was noted with reduced motor activity on study day 3, and one animal treated for 20 days was noted on study day 3 with reduced motor activity and wasted appearance.

C. BODY WEIGHT

In animals treated for three days, at 800 mg/kg bw/day that was a marked reduction in body weight and body weight gain, with a body weight loss over the three days of treatment. At 400 mg/kg bw/day, there was a slight but not statistically significant reduction in body weight and body weight gain. There was no effect of estradiol benzoate on body weight or body weight gain.

Table 5.8.3/02-1: Body weight and cumulative body weight gain in female rats administered BCS-CN88460 or estradiol benzoate for three days

	BCS-CN88460, dose in mg/kg bw/day			Estradiol benzoate
Day	**0**	**400**	**800**	**0.08 mg/kg bw/day**
1	39.8	39.1	38.1	39.8
	43.5	38.7	35.7*	42.1
3	47.8	43.7	36.8**	47.2
Gain, days 1-3, g	8.0	4.6	-1.3**	7.5

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

In animals treated for 20 days, at 800 mg/kg bw/day, there was a decrease in body weight relative to controls throughout the study, with body weight gain reduced as well although this

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decrease was not statistically significant. At 400 mg/kg w/day, there was a transient reduction on study days 2 and 3, but body weight at the end of treatment as well as total body weight gain were similar to controls.

Table 5.8.3/02-2: Body weight and cumulative body weight gain in female rats administered BCS-CN88460 for 20 days

	BCS-CN88460, dose in mgkg bw/day		
Day	**0**	**400**	**800**
1	40.0	39.7	39.0
2	42.9	39.9	37.0
3	48.5	44.9	37.9**
4	52.3	49.7	40.9
5	57.3	54.1	50.0
6	61.9	59.4	54.3
7	67.2	63.8	57.4
14	107.3	102.8	94.4
20	145.9	149.1	135.4
Gain, days 1-20, g	105.8	109.4	95.3

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

D. VAGINAL OPENING

In animals administered BCS-CN88460 for three days, the dose of 800 mg/kg bw/day exceeded the maximum tolerated dose (MTD) based on severe clinical signs and marked effects on body weight and body weight gain. Vaginal opening data were thus not collected in this group. At 400 mg/kg bw/day, there was no effect of treatment with BCS-CN88460 on vaginal opening.

In animals treated for 20 days with BCS-CN88460, it was likewise clear that the dose of 800 mg/kg bw/day exceeded the MTD, and this group was not used for determination of an effect of treatment on vaginal opening. At 400 mg/kg bw/day, there was no effect on vaginal opening.

Table 5.8.3/02-4: Mean age and body weight at vaginal opening in female rats administered BCS-CN88460 for 20 days

	BCS-CN88460, dose in mg/kg bw/day	
	0	**400**
Mean age, days	33.17	33.50
Mean body weight, g	115.17	111.22

E. NECROPSY

Mean terminal body weight was reduced at both 400 mg/kg bw/day (not statistically significant) and 800 mg/kg bw/day (statistically significant), after three days administration of BCS-CN88460. There was no effect of treatment at either dose on either wet or blotted uterine weight. Administration of estradiol benzoate at 0.08 mg/kg bw/day significantly increased uterine weight.

Table 5.8.3/02-4: Terminal body weight and uterine weight in female rats administered either BCS-CN88460 or estradiol benzoate for three days

		BCS-CN88460, mg/kg bw/day			Estradiol benzoate
		0	**400**	**800**	**0.08 mg/kg bw/day**
Terminal body wt, g		53.4	48.7	43.0**	50.7
Wet uterine weight	Absolute, mg	0.0263	0.0267	0.0260	0.0540**
	% body wt	0.04918	0.05494	0.06146	0.10825*
Blotted uterine wt	Absolute, mg	0.0244	0.0242	0.0233	0.0419*
	% body wt	0.04563	0.04979	0.05511	0.08474*

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

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After administration of BCS-CN88460 for 20 days at either 400 or 800 mg/kg bw/day, terminal body weight was only reduced (not statistically significant) at 800 mg/kg bw/day. Neither wet nor blotted absolute or relative uterine weight was increased relative to controls, although both absolute and relative liver weights were increased in both treatment groups.

Table 5.8.3/02-5: Terminal body weight and uterine weight in female rats administered BCS-CN88460 for 20 days

		BCS-CN88460, dose in mg/kg bw/day		
		0	400	800
Terminal body wt, g		150.7	153.2	140.6
Liver weight	Absolute, g	6.99	8.49*	8.12
	% body wt	4.440	5.521**	5.753**
Wet uterine weight	Absolute, mg	0.2580	0.3049	0.1933
	% body wt	0.17074	0.20154	0.13950
Blotted uterine weight	Absolute, mg	0.2453	0.2545	0.1866
	% body wt	0.16234	0.16746	0.13466

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

F. DEFICIENCIES

There are no deficiencies to report.

III. CONCLUSIONS

The administration of BCS-CN88460 to immature female rats for either three or 20 days did not alter either vaginal opening (only determined at 400 mg/kg bw/day due to exceedance of the MTD at 800 mg/kg bw/day), nor was there any effect of treatment on either wet or blotted uterine weight. Based on these results, BCS-CN88460 does not have an estrogenic effect in this assay.

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CA 5.9 Medical data

Report:	KCA 5.9/01; [redacted]; 2017; M-601949-01-1
Title:	Summary of medical data known for isoflucypram
Report No.:	M-601949-01-1
Document No.:	M-601949-01-1
Guideline(s):	Regulation (EC) No 1107/2009 & Regulation (EU) No 283/2013 Section 5: Toxicological and Metabolism studies Annex Points 5.9.1 - 5.9.7
Guideline deviation(s):	--
GLP/GEP:	**no**

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Chemical name (CAS): 1255734-28-1

Chemical name (IUPAC): *N*-(5-chloro-2-isopropylbenzyl)-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methylpyrazole-4-carboxamide

Common name: Isoflucypram

Physical state: white powder

Piloting plant(s): Bayer AG, [redacted], [redacted], Germany

Summary of in-company experience for Bayer AG, [redacted], [redacted], Germany

No medical problems related to handling of isoflucypram during the piloting phase have been reported to plant and HSE management. There is no production ongoing so far.

Occupational Medical Experiences for Bayer AG, [redacted], [redacted], Germany

All plant employees undergo annual medical examinations. There have been no specific examinations related to isoflucypram handling.

The routine annual examinations for all workers comprise:

Commenced on: 2002

Examination intervals: annually

Extent: health and work history, clinical examinations

Laboratory examinations: FBC, liver enzymes, creatinine, urine stix

Technical examinations: If indicated by work tasks,

- Spirometry
- Audiogram
- ECG, and above 35 years of age a stress test every 5 years

Other technical details: n/a

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Medical assessment for Bayer AG, [redacted], Germany

No problems were reported to the site Medical Department during the piloting phase.

During the piloting phase, no accidents with isoflucypram occurred in the workers, and no consultations of the Medical Provider due to work or contact with isoflucypram were required.

CA 5.9.2 Data collected on humans

There are no publications on human poisoning cases.

CA 5.9.3 Direct observations

No poisoning cases have come to the attention of Bayer AG, CropScience Division.

CA 5.9.4 Epidemiological studies

There are no epidemiological studies published.

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

No human cases have been reported.

In test animals, no specific signs of exposure have been seen.

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

First aid:

Remove patient from exposure / terminate exposure

- Thorough skin decontamination with copious amounts of water and soap, if available with polyethylene glycol 300 followed by water.

  Note: most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethylene glycol 300 is not required.

- Flushing of the eyes with lukewarm water for 15 minutes.

- Induction of vomiting does not seem to be required. It should only be considered if a very large amount has been swallowed, if the ingestion was less than one hour ago, and if the patient is fully conscious.

  Induced vomiting can remove maximum 50% of the ingested substance.

  Note: Induction of vomiting is forbidden if a formulation containing organic solvents has been ingested!

Treatment:

- Gastric lavage does not seem to be required in regard of the low toxicity.

- The application of activated charcoal and sodium sulphate (or other cathartic) may be considered in significant ingestions.

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- As there is no antidote, treatment has to be symptomatic and supportive.

CA 5.9.7 Expected effects of poisoning

No delayed or persisting effects are to be expected.

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APPENDIX 1 PROPOSED TOXICOLOGICAL CLASSIFICATION OF ISOFLUCYPRAM

This appendix provides a detailed comparison of potentially classification-relevant toxicological findings of isoflucypram with the respective applicable CLP criteria (following the ECHA Guidance to Regulation (EC) No. 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 5 of July 2017). As an outcome of this exercise, proposals for classification / non-classification are made for acute toxicity, skin irritation, eye irritation, skin sensitization, germ cell mutagenicity, carcinogenicity, reproductive toxicity, STOT-SE, STOR-RE.

ACUTE TOXICITY, SKIN IRRITATION, EYE IRRITATION, SKIN SENSITIZATION

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling, and packaging (CLP) of substances and mixtures, Version 5.0 of July 2017, the results of the acute toxicological studies conducted with isoflucypram are:

Study	Result	Reference
Oral LD50 rat	> 2000 mg/kg bw	M-485872-01-1
Dermal LD50 rat	> 2000 mg/kg bw	M-485659-01-1
Inhalation LC50 rat	> 2.518 mg/L both sexes combined; 3.131 mg/L in males, 2.209 mg/L in females	M-502440-01-1
Skin irritation	negative	M-484711-01-1
Eye irritation	negative	M-493768-01-1
Skin sensitization	positive in the mouse LLNA	M-524452-01-1

The acute inhalation toxicity study, with an LC50 of 2.518 mg/L in both sexes combined, triggers classification for acute inhalation toxicity. As the LC50 is between 1.0 and 5.0 mg/L (Table 3.1.1 of Annex 1 to the Regulation (EC) No. 1272/2008), isoflucypram must be classified for inhalation toxicity (Cat. 4).

In the mouse local lymph node assay, the Stimulation Index increased in a dose-related manner and exceeded 3.0 at concentrations of 25% and 50% (the top concentration used). The EC3 in this study was 29%. Isoflucypram is thus sensitizing in the mouse local lymph node assay, which requires classification as a weak sensitizer (Cat. 1B).

Conclusion on classification and labelling:

CLP Regulation: H332 (Harmful if inhaled) and H317 (May cause an allergic skin reaction)

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GENOTOXICITY

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 5.0 of July 2017, a classification for germ cell mutagenicity Category 2 is based on

- Positive evidence obtained from experiments in mammals and / or in some cases from *in vitro* experiments, obtained from:
  - Somatic cell mutagenicity tests *in vivo*, in mammals; or
  - Other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays.
  -

An additional note is provided: Substances which are positive in *in vitro* mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2 mutagens.

A battery of genotoxicity studies conducted with isoflucypram shows the following results (see also Table 5.4-1 in this MCA Summary):

Study	Result		Reference
	+ S9	- S9	
Bacterial mutation assay (Ames test) in S. typhimurium	Negative	Negative	M-490251-01-1
Chromosome aberration *in vitro* (human lymphocytes)	Positive	Positive	M-495533-01-1
Mammalian cell gene mutation *in vitro* (V79 hamster cells)	Negative	Negative	M-488526-01-1
Micronucleus assay *in vivo* (mouse bone marrow)	Negative	Not relevant	M-485866-01-1

Isoflucypram did not increase the incidence of reverse mutations in the Ames assay, nor did it show any mutagenic potential in mammalian cells *in vitro*.

In the *in vitro* chromosomal aberrations assay in human lymphocytes, both in the absence and the presence of S9 metabolic activating system, there was an increase in the incidence of structural chromosomal aberrations, both when gaps were included and when they were excluded. There was no increase in the incidence of exchanges noted, nor was there an increase in the incidence of polyploidy under any experimental conditions tested.

In the mouse micronucleus assay *in vivo*, clinical signs of toxicity were noted (reduction of spontaneous activity, apathy or excitement, abdominal position) after oral gavage administration of isoflucypram to mice. There was no alteration in the ratio of polychromatic to normochromatic erythrocytes, however ADME studies in the rat (see Point 5.1 in this MCA Summary) showed that isoflucypram reaches the bone marrow after oral gavage administration, and thus the bone marrow will have been reached in the mouse micronucleus study. There was no increase in the incidence of micronucleated polychromatic erythrocytes, and thus the *in vivo* mouse micronucleus study is negative.

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Thus, although isoflucypram was positive in an *in vitro* test for genotoxicity, this *in vitro* result was not confirmed by the relevant *in vivo* study. Isoflucypram does not have any structural relationship to known germ cell mutagens.

Conclusion on classification and labelling:

It is concluded that no classification for germ cell mutagenicity is applicable for isoflucypram according to the above-mentioned ECHA Guidance.

CLP regulation: No classification.

CARCINOGENICITY

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling, and packaging (CLP) of substances and mixtures, version 5.0 of July 2017, classification for carcinogenicity requires that exposure to the test substance increases the incidence of neoplasms.

Isoflucypram was tested for carcinogenicity in the rat ([redacted]; 2018; M-612739-02-1) and in the mouse ([redacted]; 2017; M-593645-01-1). In the rat, concerns for increased mortality due to potential male rat-specific nephrotoxicity caused selection of doses which did not in fact evoke marked toxicity in either the males (highest dose tested: 18.6 mg/kg bw/day) or females (46.6 mg/kg bw/day). In the mouse, isoflucypram was tested at up to 1250 ppm (147 / 190 mg/kg bw/day in males / females). At the highest dietary concentration of 1250 ppm, there was a marginal increase in mortality in females, and body weight gain was decreased in both males (by 11%) and females (by 13%). Liver weights were increased, and the incidence of multinucleated hepatocytes and single-cell necrosis was increased in males, while the incidence of bile duct hyperplasia and hepatocellular necrotic foci was increased in females. There were no neoplastic findings in any organ in either males or females, and thus no evidence of a tumorigenic or carcinogenic potential was observed in the mouse 18-month study.

Conclusion on classification and labelling:

It is therefore concluded that isoflucypram is not carcinogenic and that classification for carcinogenicity is not warranted.

CLP regulation: No classification.

REPRODUCTIVE TOXICITY

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling, and packaging (CLP) of substances and mixtures, version 5.0 of July 2017, substances must be examined for classification for adverse effects on sexual function and fertility.

In a two-generation study ([redacted], R.; 2018; M-612750-02-1), Wistar rats were administered isoflucypram via the diet at concentrations of up to 1200 ppm in males and during the pre-mating, mating and gestation phases of the study in females, and of 600 ppm in females during lactation. The dietary concentrations were reduced in all treatment groups during the lactation phase to maintain the systemic dose at a relatively constant level and to avoid over-dosing the offspring as they began to consume diet prior to actual weaning. There were no treatment-related effects on body weight, body weight gain, or any endpoints related to fertility, fecundity, or development. Liver weight was increased in both adults and offspring, in a dose-related manner. This increase in liver weight is considered related to the induction of hepatic Phase I and Phase II enzymes and hepatocellular

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hypertrophy as observed in short-term studies (see also Points 5.3.1/01 and 5.3.2/01 in this MCA Summary).

Thus, in this study on the potential reproductive effects of isoflucypram on successive generations in the rat, there were no effects on any endpoints related to either reproduction or development.

Conclusion on classification and labelling:

Isoflucypram did not produce any effects on fertility, fecundity, or development in the 2-generation rat study.

Classification of isoflucypram for reproductive toxicity is therefore not justified.

CLP regulation: No classification.

DEVELOPMENTAL TOXICITY

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling, and packaging (CLP) of substances and mixtures, version 5.0 of July 2017, substances must be examined for classification based on their potential effects on development of the offspring. As stated in the Guidance, the major manifestations of developmental toxicity include:

- Death of the developing organism,
- Structural abnormality,
- Altered growth, and
- Functional deficiency.

In the rat developmental toxicity study ([redacted], 2017; M-602126-01-1), isoflucypram was administered to pregnant rats at doses of 0, 25, 125, and 625 mg/kg bw/day. At the top dose, maternal body weight gain was markedly reduced during the first two days of the treatment period, and corrected body weight change was reduced by 10% compared to controls. Liver weight was increased, the macroscopic finding "enlarged liver" was observed in 14/23 animals, and the incidence of centrilobular hypertrophy was observed in all animals subjected to histopathological examination. At 625 mg/kg bw/day, fetal body weight was slightly reduced compared to controls, and there was a marginal retardation of ossification. There were no fetal findings at 125 mg/kg bw/day. Thus, the maternal and fetal NOAEL was set at 125 mg/kg bw/day. There was no evidence of a teratogenic effect.

In the rabbit developmental toxicity study ([redacted]; 2017; M-588469-01-1), isoflucypram was administered to pregnant rabbits at doses of 0, 10, 70, and 500 mg/kg bw/day. At the top dose, maternal body weight gain and corrected body weight change were reduced compared to controls, and food consumption was decreased between gestation days 6 and 14. Liver weight was increased compared to the control group. There were no fetal findings in any treatment group. Thus, the maternal NOAEL was set at 70 mg/kg bw/day, while the fetal NOEL was 500 mg/kg bw/day.

With regard to death of the developing organism, there was no increase in fetal mortality in either the rat or the rabbit developmental toxicity studies, nor was there an increase in fetal or offspring loss in the 2-generation reproductive toxicity study.

Structural abnormalities at the external, visceral, or skeletal level were not seen in either the rat or the rabbit developmental toxicity studies.

Altered growth of the fetuses was not evident in either developmental toxicity study. The marginal delays in ossification in the rat developmental toxicity study are reflective of the slight decrease in fetal body weight, which in turn is secondary to the decreased maternal body weight gain in that same group.

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Functional deficiency cannot be assessed in developmental toxicity studies, although there were no structural or developmental findings which would have indicated a functional deficiency in the fetuses. Administration of isoflucypram to adult female rats during pre-mating, mating, gestation, and lactation as well as to the offspring during their own pre-mating, mating, gestation, and lactation periods had no effect on the function including reproductive function of the female rat. Likewise, fertility of male rats was not affected in the 2-generation reproduction study. Thus, it can be concluded that neither dietary nor oral gavage administration of isoflucypram to pregnant animals caused functional deficiences in the offspring.

Conclusion on classification and labelling:

Thus, it can be concluded that isoflucypram is neither teratogenic nor developmentally toxic, and classification of isoflucypram for developmental toxicity is not justified.

CLP regulation: No classification.

SPECIFIC TARGET ORGAN TOXICITY – SINGLE EXPOSURE (STOT-SE)

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling, and packaging (CLP) of substances and mixtures, version 5.0 of July 2017, substances are to be classified if they cause specific, non-lethal target organ toxicities resulting from single exposures to the substance. In cases where a single exposure to a substance causes lethality, that effect should result in classification for acute toxicity, not for STOT-SE.

The mortality observed in the acute inhalation study is thus covered under the classification of isoflucypram for Acute Toxicity, Category 4, H332 (Harmful if inhaled).

Classification of a substance for STOT-SE, Category 2, is not applicable if non-lethal significant and / or severe toxic effects are not seen in acute toxicity studies up to 2000 mg/kg bw after either oral or dermal exposure, or up 5 mg/L/4h after inhalation exposure.

The ECHA Guidance also specifies criteria that trigger a classification for STOT-SE Category 3, which are generally independent from the aforementioned guidance values and include transient target organ effects including overt narcotic effects and respiratory tract irritation.

Studies in which the acute effects of isoflucypram were examined, the LOAELs, and the toxicological effects at the LOAELs, are presented in the following table.

Study Reference	LOAEL	Findings at LOAEL
Acute oral rat M-485872-01-1	> 2000 mg/kg bw (highest dose tested)	No clinical signs, no findings
Acute dermal rat M-485659-01-1	> 2000 mg/kg bw/ (highest dose tested)	No clinical signs, no findings
Acute inhalation rat M-502440-01-1	1.03 mg/L	On the day of exposure: labored, noisy, or gasping respiration. No clinical signs were noted beyond the day of exposure.
Acute neurotoxicity rat M-594177-01-1	> 2000 mg/kg bw/day (highest dose tested)	No clinical signs, no findings, no neurotoxicity

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A comparison of these LOAELs and toxicological effects with the criteria for classification shows that classification of isoflucypram with STOT-SE Category 2 is not justified.

Regarding a possible STOT-SE Category 3 classification for either respiratory tract irritation or narcotic effects, there was no indication in the acute inhalation study of irritation of the respiratory tract. Furthermore, the absence of any decrease in motor activity or other effects in the acute neurotoxicity study indicates that isoflucypram does not have a narcotic effect after acute administration. Thus, a STOT-SE Category 3 classification is not justified.

Conclusion on classification and labelling:

It can be concluded that classification of isoflucypram for STOT-SE is not warranted based on the effects observed after acute exposure to the substance.

CLP regulation: No classification.

SPECIFIC TARGET ORGAN TOXICITY – REPEATED EXPOSURE (STOT-RE)

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling, and packaging (CLP) of substances and mixtures, version 5.0 of July 2017, classification in STOT-RE Category 2 is not applicable if significant toxic effects are not observed in 28- or 90-day or 12-month repeated exposure studies in experimental animals up to the following guidance values:

Exposure route	28-day	90-day	12-month	> 12 month
Oral	300 mg/kg bw/day	100 mg/kg bw/day	25 mg/kg bw/day	No guidance value
Dermal	600 mg/kg bw/day	200 mg/kg bw/day	No guidance value	No guidance value
Inhalation	600 mg/m3	200 mg/m3	No guidance value	No guidance value

As repeat-dose studies were not conducted by either the dermal or the inhalation routes, these trigger values are not relevant for the current case.

In studies conducted with isoflucypram, the findings observed in repeat-dose studies are summarized in the table below with the LOAEL of each study.

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Study	LOAEL (mg/kg bw/day)	Effects at LOAEL
28-day rat M-464024-03-1	83.3 / 86.5 males / females	Increased liver weight, hepatocellular hypertrophy; increased thyroid weight, follicular cell hypertrophy; induction of specific hepatic Phase I and Phase II enzymes
28-day mouse M-442490-01-1	133 / 374 males / females	Increased liver weight, hepatocellular hypertrophy, hepatocellular necrotic foci, single cell necrosis, increased ASAT, ALAT, AP
28-day dog M-503716-01-1	76.9 / 90.2 males / females	Decreased body weight, body weight gain, increased liver weight, hepatocellular hypertrophy
90-day rat M-487478-02-1	63.5 / 80.9 males / females	Increased liver weight, hepatocellular hypertrophy, cellular casts in urine, hyaline droplets, male-rat-specific alpha2u-globulin accumulation
90-day mouse M-472773-01-1	168 / 207 males / females	Increased liver weight, mainly centrilobular hepatocellular vacuolation
90-day dog M-520001-01-1	50.4 / 54.0 males / females	Increased liver weight, alkaline phosphatase, hepatocellular hypertrophy
One-year dog M-601188-01-1	18.8 / 17.6 males / females	Increased liver weight, alkaline phosphatase, hepatocellular hypertrophy
Chronic rat (12-month timepoint) M-612739-02-1	> 21.3 / > 52.1 males / females	No LOAEL

From the data above, the primary target organ of isoflucypram is the liver in the rat, mouse, and dog. The thyroid is also affected in the rat, although this is secondary to a CAR/PXR-mediated mode of action and is therefore specific to the rat. Similarly, the effects observed in the kidney of the male rat are precursors of the male rat-specific chronic progressive nephropathy and are not relevant for human risk assessment.

The LOAELs in the studies tabulated above are below the trigger values for STOT-RE classification, and thus isoflucypram is to be classified STOT-RE Category 2 for its effects in the liver.

Conclusion on classification and labelling:

Based on the effects observed in short-term studies, isoflucypram is to be classified STOT-RE, Category 2 for its effects in the liver.

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APPENDIX 2 ENDPOINTS RELEVANT FOR RISK ASSESSMENT OF ISOFLUCYPRAM

In the subacute, subchronic, and chronic studies conducted with isoflucypram, the liver was the target organ in rat, mouse, and dog. The kidney was a target organ in the rat studies as well as in the mouse chronic study, while the thyroid was a target organ only in the rat.

In the **rat 28-day study (data point 5.3.1/01)**, body weight was reduced in males but not in females, while the absolute and relative weights of liver and thyroid were increased in both sexes. Treatment-related histopathological findings were observed in the liver (hepatocellular hypertrophy) and thyroid (follicular cell hypertrophy) of both sexes and the kidney (tubular hyaline droplets, bilateral basophilic tubules) of males only. Cholesterol concentrations were increased and total bilirubin concentrations were decreased in a dose-related manner, suggesting that isoflucypram was acting through a CAR-PXR mode of action. Isoflucypram was shown to induce specific cytochrome P450 isozymes and Phase II conjugating enzymes which are known to be linked to the CAR-PXR mode of action. The NOAEL of the rat 28-day study was 300 ppm (22.8 mg/kg bw/day in males and 25.6 mg/kg bw/day in females). The LOAEL was 1000 ppm (83.3 mg/kg bw/day in males and 86.5 mg/kg bw/day in females).

In the **rat 90-day study (data point 5.3.2/01)**, body weight and body weight gain were slightly reduced in both males and females. Mean terminal body weight was slightly reduced in both sexes, and absolute and / or relative liver weights were increased with a greater effect in females than in males. Relative thyroid and kidney weight were increased in males only. Treatment-related histopathological findings were observed in the liver (hepatocellular hypertrophy), thyroid (follicular cell hypertrophy, colloid alteration), and in the males in kidney (hyaline droplets, bilateral basophilic tubules). Total bilirubin was decreased, and in males urinalysis showed cellular casts at the top dose. The NOAEL of the rat 90-day study was 300 ppm (18.4 mg/kg bw/day in males and 21.9 mg/kg bw/day in females). The LOAEL was 1000 ppm (63.4 mg/kg bw/day in males and 80.9 mg/kg bw/day in females).

In the **rat 2-year study (data point 5.5/01)**, there was a very slight decrease in body weight and body weight gain. Total bilirubin was slightly decreased in females. Absolute and relative liver weights were slightly increased in females. Treatment-related histopathological findings were observed only in the thyroid, the incidence and severity of colloid alteration was increased in both sexes, while the incidence of diffuse pigmentation of the follicular cells was only increased in males. The NOAEL of this study was greater than the highest dose tested, 450 ppm (18.6 mg/kg bw/day) in males and 800 ppm (46.6 mg/kg bw/day) in females.

In the **mouse 28-day study (data point 5.3.1/02)**, absolute and relative liver weights were increased in both males and females. Treatment-related histopathological findings were observed in the liver (hepatocellular hypertrophy and hepatocellular necrotic foci) in both sexes. Aspartate aminotransferase and alkaline phosphatase were increased, and total bilirubin concentrations were decreased in a dose-related manner in females only. The NOAEL of the mouse 28-day study was 800 ppm (approximately 149 mg/kg bw/day) in males and 200 ppm (approximately 32 mg/kg bw/day) in females. The LOAEL was 2000 ppm (330 mg/kg bw/day) in males and 800 ppm (149 mg/kg bw/day) in females.

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In the **mouse 90-day study (data point 5.3.2/02)**, absolute and relative liver weights were increased in both males and females, and were accompanied by an increase in the incidence of diffuse mainly centrilobular hepatocellular vacuolation and a loss of vacuolation in the periportal regions. Total bilirubin was decreased in both males and females. The NOAEL in the mouse 90-day study was 300 ppm (51.0 mg/kg bw/day in males and 59.8 mg/kg bw/day in females). The LOAEL was 1000 ppm (168 mg/kg bw/day in males and 207 mg/kg bw/day in females).

In the **mouse 18-month study (data point 5.5/02)**, body weight and body weight gain were reduced at the top dose, and terminal body weight was decreased at the terminal sacrifice in both sexes. Absolute and relative liver weights were increased in males, accompanied by an increased incidence of multinucleated hepatocytes and single cell necrosis; in females liver weight was not affected but histopathological findings included an increase in the incidence of diffuse bile duct hyperplasia and of hepatocellular necrotic foci, and a decrease in the incidence and severity of diffuse hepatocellular vacuolation. Absolute and relative kidney weight were also increased in males only, accompanied by an increase in the incidence of hyaline casts, tubular dilation in the medulla and a slightly higher severity of focal tubule basophilia. The NOAEL of the 18-month mouse study was 250 ppm (29.0 mg/kg bw/day in males and 38.1 mg/kg bw/day in females). The LOAEL was 1250 ppm (147 mg/kg bw/day in males and 190 mg/kg bw/day in females.

In the **dog 28-day study (data point 5.3.1/03)**, body weight was decreased in three of four animals at the top dose, and terminal body weight was decreased in females at the top dose. Alkaline phosphatase was increased in three of the four animals at the top dose. Absolute and relative liver weights were increased in both males and females, accompanied by hepatocellular hypertrophy. The NOAEL in the dog 28-day study was 1000 ppm (37.7 mg/kg bw/day in males and 36.5 mg/kg bw/day in females). The LOAEL was 3000 ppm (76.9 mg/kg bw/day in males and 90.2 mg/kg bw/day in females).

In the **dog 90-day study (data point 5.3.2/03)**, body weight was slightly reduced and body weight gain was reduced in both males and females. Alkaline phosphatase activity was increased in both males and females, and total bilirubin was decreased in both sexes. Terminal body weight was slightly reduced, while absolute and relative liver weights were increased. Increased liver weight was accompanied by hepatocellular hypertrophy in males only. The NOAEL in the dog 90-day study was 500 ppm (15.9 mg/kg bw/day in males, 16.2 mg/kg bw/day in females).

In the **dog one-year study (data point 5.3.2/04)**, body weight and / or body weight gain were slightly reduced in both sexes at various points during the study. Food consumption was reduced in females throughout the study. Alkaline phosphatase was increased at various time points, although these increases were generally not statistically significant except at the top dose. Absolute and relative liver weights were increased in males, but not in females, although both sexes showed hepatocellular hypertrophy at the high dose. The NOAEL in the dog one-year study was 150 ppm (4.2 mg/kg bw/day in both sexes). The LOAEL was 600 ppm (18.8 mg/kg bw/day in males and 17.6 mg/kg bw/day in females).

In the **rat 2-generation reproduction study (data point 5.6.1/01)**, there were no treatment-related effects on any reproductive parameters including fertility, fecundity, or sexual maturation. Liver weight was increased in a dose-related manner in both adults and offspring. The NOAEL for reproductive performance of the F0 and F1 adults, and the NOAEL for the survival, growth, and development of the F1 and F2 offspring, is considered to be 1200 ppm for adult males (94.4 and 108.6 mg/kg bw/day in F0 and F1 adult males, respectively) and 1200 / 600 ppm for adult females and for offspring (from 92.9 to 140.4 mg/kg bw/day depending on generation and phase of the study).

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In the **rat developmental toxicity study (data point 5.6.2/01)**, maternal body weight gain between gestation days 6 and 8 was decreased, and overall mean body weight gain and corrected body weight change were slightly decreased at the end of the study. Liver weight was increased. At histopathology, treatment-related changes were increased incidence of hepatocellular hypertrophy and thyroid follicular cell hypertrophy. Fetal body weight was slightly decreased relative to controls. At fetal examination, there was an increase in the incidence of distended bladder, renal pelvis dilatation, and delayed or incomplete ossification of a number of skeletal structures. The NOAEL of this study was 125 mg/kg bw/day for both maternal and fetal toxicity. The LOAEL was 625 mg/kg bw/day.

In the **rabbit developmental toxicity study (data point 5.6.2/02)**, maternal body weight decreased between gestation days 6 and 8, followed by slight decreases in body weight gain and a decrease in overall corrected body weight change. Liver weight was increased relative to controls. Additionally, two dams in the top dose group aborted, one on gestation day 23 and one on gestation day 26. There were no effects on fetal body weight, or on external, visceral, or skeletal findings. The maternal NOAEL for this study was 70 mg/kg bw/day, while the fetal NOEL was the top dose of 500 mg/kg bw/day. The maternal LOAEL was 500 mg/kg bw/day, and the fetal LOAEL was greater than the top dose of 500 mg/kg bw/day.

ADI

Based on the findings briefly summarized above the rat shows a greater response to dietary administration of isoflucypram than does either the mouse or the dog. The liver is the target organ in all three species however in the rat the kidney (in males only) and the thyroid are also affected after subacute and subchronic administration. Although the kidney effects observed in the male rat are related to the exacerbation of male rat-specific chronic progressive nephropathy (data points 5.3.1/01 and 5.3.2/01), the observation of kidney findings in the male mouse suggests that kidney observations cannot be completely overlooked.

The use of the male rat for derivation of the risk assessment endpoints is therefore more protective than use of either the mouse or the dog. The NOEL of the rat chronic study, 6.3 mg/kg bw/day, will therefore be used for derivation of the ADI, with standard safety factors of 100, resulting in an ADI of 0.063 mg/kg bw/day.

Conclusion for ADI-proposal: 0.063 mg/kg bw/day

ARfD

Isoflucypram has essentially no systemic toxicity after acute administration. The majority of the effects observed in repeat-dose studies (alterations in liver, kidney, and / or thyroid weight and histopathology) are indicative of an adaptation to dietary administration of the compound. Frequently, developmental toxicity studies are used for derivation of the acute risk assessment endpoints such as the Acute Reference Dose and the Acute AOEL due to the possibility for irreversible developmental effects occurring from only one exposure to a toxicant.

Thus, the developmental toxicity studies will be examined for use in derivation of the ARfD. Because both maternal and fetal findings were observed in the rat developmental toxicity study, while no fetal findings were seen at any dose in the rabbit developmental toxicity study, the rat study will be used for derivation of the ARfD.

Using the NOAEL of 125 mg/kg bw/day from the rat developmental toxicity study, and standard uncertainty factors of 100, the ARfD is 1.25 mg/kg bw/day.

Conclusion for ARfD-proposal: 1.25 mg/kg bw/day

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AOEL

As described earlier, use of the rat for derivation of systemic endpoints is more conservative than use of other species. Thus, the NOAEL of 18.4 mg/kg bw/day from the rat 90-day study will be used with uncertainty factors of 100 to derive a systemic AOEL of 0.18 mg/kg bw/day.

Conclusion for AOEL-proposal: 0.18 mg/kg bw/day

AAOEL

As described for derivation of the ARfD, in the absence of acute toxicity, the developmental toxicity studies can be used to derive acute risk assessment endpoints. Because both maternal and fetal findings were observed in the rat developmental toxicity study, while no fetal findings were seen at any dose in the rabbit developmental toxicity study, the rat study will be used for derivation of the AAOEL.

Based on the NOAEL of 125 mg/kg bw/day in the rat developmental toxicity study, and standard uncertainty factors of 100, the systemic AAOEL is 1.25 mg/kg bw/day.

Conclusion for AAOEL-proposal: 1.25 mg/kg bw/day

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